Mitochondrial membrane depolarization and caspase dependent apoptosis induced by Cannabis sativa

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ABSTRACT

The anticancer effect of Cannabis sativa leaf ethanolic extract (CSL-E) and its n-hexane fraction (CSL-F) was evaluated against a panel of human cancer cell lines including colon carcinoma Colo-205, prostate adenocarcinoma PC-3, lung carcinoma A-549 and promyelocytic leukemia HL-60. CSL-E and CSL-F inhibited the growth of cell lines in a dose-dependent manner with IC50 values varied from 32.51 - 89.28 and 25.71 - 56.26 µg/ml respectively depending on the cell line. In addition, CSL-F induced concentration dependent apoptosis in HL-60 cells as measured by various endpoints, e.g. annexin V binding, DNA fragmentation, apoptotic bodies' formation and an increase in hypodiploid subG0 DNA content. Moreover, translocation of Bax to mitochondria leads to loss of mitochondrial membrane potential and release of cytochrome c into the cytosol. These events were associated with significant activation of caspase-3, -6 and -9 which further led to Poly (ADP-ribose) polymerase cleavage. Taken together, these results demonstrated that CSL-F inhibits the proliferation of cancer cells through induction of apoptosis, making CSL-F a candidate for anticancer therapy.

Key words: Cannabis sativa, Apoptosis, Mitochondrial membrane potential, Cytochrome c, Caspase.

INTRODUCTION

Malignant cancer is the second leading cause of death worldwide. More than 75% of anti-cancer drugs are directly or indirectly derived from medicinal plants but still there is a continuing need for development of new anticancer drugs, drug combinations and chemotherapy strategies, through scientific exploration of the enormous pool of natural products. Recent reports have claimed that many anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent tumor promotion and progression. Mitochondria are currently regarded as playing a central role in mediating ‘intrinsic death signals’ and could serve as a novel target for chemotherapeutics. In our efforts towards the development of novel herbal products for their anti-cancer potential, we report here to the best of our knowledge for the first time, the pro-apoptotic effect of n-hexane fraction (CSL-F) of Cannabis sativa leaves and its usefulness in the development of anticancer therapeutic leads.

Cannabis sativa is an annual plant in the Cannabaceae family, commonly known as marijuana. It is an herb that has been used throughout recorded history by humans for various purposes such as fiber, oil, food, drug, medicine and spiritual satisfaction. The use of cannabis as a medicine is increasingly becoming a topic of public discussion in a growing number of countries around the world. Although THC is known to be the major active compound in the cannabis plant, it is widely believed by researchers that other components, predominantly the cannabinoids also could play a role in the medicinal properties of cannabis.

In this report, we investigate the mechanism of CSL-F on the induction of apoptosis effect in human leukemia HL-60 cells. In order to evaluate whether the anti-proliferative effect of CSL-F in HL-60 cells was mediated through apoptosis, we came to conclude from the results that CSL-F can indeed promote apoptosis in HL-60 cells via mitochondrial membrane depolarization and activation of the caspase cascade. Further studies are in the pipeline to isolate essential molecule(s) for the development of innovative therapeutic agent against cancer.

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MATERIAL AND METHODS

Chemicals and antibodies
RPMI-1640 medium, rhodamine-123 (Rh-123), propidium iodide (PI), sulforhodamine B (SRB), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), DNase-free RNase, proteinase K, phenylmethanesulfonyl fluoride (PMSF), eukaryotic protease inhibitor cocktail, Triton X-100, penicillin, streptomycin, L-glutamine, pyruvic acid, camptothecin, Fetal bovine serum and AnnexinV-FITC apoptosis detection kit were purchased from Sigma Aldrich, USA. Tris-Base, EDTA and Phosphate buffered saline (PBS) were purchased from HiMedia Laboratories Pvt. Ltd. India and Trichloroacetic acid was from Merck Specialties Pvt. Ltd. India. Caspase-3, -6, and -9 assay kits were from BioVision, Inc. USA. FITC mouse anti-cleaved PARP (ASP214) antibodies and Mouse anti-human antibodies to cytochrome c were from BD Biosciences, Pharmingen, USA. Mouse anti-human antibodies to Bax, b-Actin, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were from Santa Cruz Bio-technology, USA. Electrophoresis reagents, Protein estimation kit and protein markers were from Bio-Rad Laboratories, USA. Hyper film and ECL Plus western blotting detection kit were from Amersham Biosciences, UK.

Plant material and extraction
Leaves of C. sativa were harvested from herbal garden of Indian Institute of Integrative Medicine (IIIM), Jammu, India. A specimen of the plant was collected and submitted to the herbarium of the Department of Botany, IIIM, Jammu, India (Accession no. 18255) as authenticated by the taxonomist, Dr. S.N. Sharma of the same department of the Institute.

Dried and powdered leaves of Cannabis sativa (500 g) were placed in a conical glass percolator, submerged with 95% ethanol and kept overnight at room temperature for 20 h. The percolate was collected and filtered. Ethanol was distilled off using rotavapour under reduced pressure at 50°C. The final drying was done initially in a vacuum desiccator and finally in lyophilizer to get dried ethanolic extract, CSL-E (yield: 11% of dry wt).

Fractionation of Cannabis sativa leaves extract was performed according to the method of Li et al. with some modifications. The dried ethanolic extract (10 g) was taken in a stoppered conical flask, vigorously shaken with 500 ml n-hexane and allowed to stand for 30 min. The supernatant was decanted, Procedure was repeated four times using fresh n-hexane every time. The combined n-hexane soluble portion was evaporated to dryness under reduced pressure below 50°C. The
dried isolate, CSL-F (yield: 65% of dry wt) was transferred to an air tight glass container. Nitrogen was blown in the container before capping and stored at -20°C in desiccator before use.

Cell culture and treatment
The human cancer cell lines were obtained from National Cancer Institute, Frederick, U.S.A. Colon (Colo-205, SW-620), ovary (OVCAR-5, IGROV-1), prostate (PC-3, DU-145), lung (A-549, Hop-62), cervix (SiHa, Hela) and leukemia (HL-60, MOLT-4) cell lines were grown and maintained in RPMI-1640 medium, pH 7.4. The media was supplemented with FCS (10%), penicillin (100 Units/ml) and streptomycin (100 µg/ml), being referred to as complete medium. The cells were grown in CO2 incubator (Hera Cell, Heraeus, Germany) at 37°C with 90% humidity and 5% CO2. Stock solution (20 mg/ml) of CSL-E and CSL-F were prepared in water and serially diluted with complete growth medium containing 50 µg/ml of gentamicin to obtain desired concentrations.

Cytotoxicity assay
The in vitro cytotoxicity against human adherent cancer cell lines was determined by SRB assay according to the method of Monks et al. as described previously. The samples were further screened in leukemia cell lines namely MOLT-4 and HL-60 using MTT assay as per standard procedure.

Light microscopy
HL-60 cells (1x10⁶/ml) were treated with CSL-F at 10 and 30 µg/ml for 24 hr. Cells were centrifuged at 1000 rpm for 5 min and resuspended in PBS. Cells were observed under phase contrast microscope and morphology was assessed and photographed.

Annexin V/propidium iodide (PI) flow cytometric analysis
Phosphatidyserine exposed on the outside of the apoptotic cells was determined by an Annexin V-FITC Apoptosis Kit (Sigma). Briefly, following treatment with CSL-F for 24 hr, HL-60 cells (5x10⁶/ml) were harvested by low speed centrifugation, washed twice with ice-cold PBS, pelleted and resuspended in 400 µl of 1 X Annexin V-FITC binding buffer, 4 µl of Annexin V-FITC conjugate and 8 µl of PI buffer. The cells were then incubated at room temperature for 15 min in the dark and analyzed using a FACS Calibur (Becton Dickinson, USA).

DNA agarose gel electrophoresis
Apoptosis was assessed by electrophoresis of extracted genomic DNA from HL-60 cells as described previously.

DNA content and cell cycle phase distribution
Changes in mitochondrial transmembrane potential (Δψm) as a result of mitochondrial perturbation were measured after staining with Rhodamine-123. HL-60 cells (5x10⁶/ml) were treated with CSL-F at 10, 30 and 100 µg/ml of CSF for 24 hr, fixed in cold 70% alcohol in PBS, washed, digested with DNase free RNase (10 µg/ml) at 37°C for 1 hr and stained with propidium iodide (PI, 5 µg/ml) for 3 hr at 4°C in dark. Cells were analyzed immediately on FACS Calibur (Becton Dickinson, USA). The fluorescence intensity of sub-G0 cell fraction represents the apoptotic cell population.

Measurement of mitochondrial membrane potential
The mitochondrial membrane potential (Δψm) of human cancer cell lines was determined by the mitochondrial membrane potential dye, Rh123. HL-60 cells (5x10⁶/ml) were treated with CSL-F for each cell line are described in Table 1. Both the treatments exhibited maximum cytotoxicity against the colon cancer cell line, Colo-205 with IC50 value of 32.51 ± 1.27 and 25.71 ± 0.86µg/ml respectively. However, the IC50 value of CSL-F was lower and hence more cytotoxic than its original aqueous extract CSL-E.

Caspase assays
HL-60 cells (2x10³/ml/well, 6-well plate) were incubated with CSL-F for the indicated time period. At the end of treatment, cells were washed in PBS and pellet lysed in cell lysis buffer. Activities of caspase-3, -6, and -9 in the cell lysates were determined colorimetrically using BioVision colorimetric caspase assay kits. Caspase-3, -6 and -9 employed chromatophore conjugated peptides DEVD-pNA, VEID-pNA and LEHD-pNA as substrates, respectively. Release of p-nitroanilide (pNA) was assayed as per the supplier’s instructions.

PARP Cleavage
HL-60 (2x10³/ml/well, 6-well plate) cells were treated with CSL-F at various concentrations for 24 hr. Cells were harvested by low speed centrifugation, washed twice with ice-cold PBS, pelleted and resuspended in 1 ml of 2 % (w/v) parafomaldehyde and incubated for 20 min on ice for fixation. Fixed cells were then permeabilized in 0.1% Triton X-100 for 30 min, washed and resuspended in 100 µl of PBS. Further the cells were incubated with 20 µl of FITC mouse anti-cleaved PARP antibody (BD Biosciences) at room temperature for another 30 min in the dark. Finally, the samples were washed twice and resuspended in 500 µl PBS and analyzed for cleaved PARP using a FACS Calibur (Becton Dickinson, USA).

Western blot analysis
The protein lysates prepared as described previously were subjected to discontinuous SDS-PAGE analysis. Proteins aliquots (50 µg) were resolved on SDS-PAGE and then electro transferred to PVDF membrane overnight at 4°C at 30 V. Non-specific binding was blocked by incubation with 5 % non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hr at room temperature. The blots were probed with respective primary antibodies for 2 hr and washed three times with TBST. The blots were then incubated with horseradish peroxidase conjugated mouse or rabbit secondary antibodies for 1 hr, washed again three times with TBST and signals detected using ECL plus chemiluminescence’s kit on X-ray film.

Statistical analysis
All experimental data were expressed as Mean ± S.E. The statistical significance of the difference between control and treated groups was determined by student’s t-test. P value: *p < 0.05 and **p < 0.01 were considered to be significant and highly significant respectively.

RESULTS

Assessment of in vitro cytotoxicity
In vitro cytotoxicity studies of CSL-E and CSL-F showed dose-dependent effect against several human cancer cell lines of different tissues such as colon, ovary, prostate, lung, cervix and leukemia (data not shown). IC50 values of CSL-E and CSL-F for each cell line are described in Table 1. Both the treatments exhibited maximum cytotoxicity against the colon cancer cell line, Colo-205 with IC50 value of 32.51 ± 1.27 and 25.71 ± 0.86µg/ml respectively. However, the IC50 value of CSL-F was lower and hence more cytotoxic than its original aqueous extract CSL-E.

Table 1. IC50 values of CSL-E and CSL-F against human cancer cell lines

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell Line</th>
<th>IC50 Value (µg/ml)</th>
<th>CSL-E</th>
<th>CSL-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>Colo-205</td>
<td>32.51 ± 1.27</td>
<td>25.71 ± 0.86</td>
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<tr>
<td></td>
<td>SW-620</td>
<td>43.30 ± 2.11</td>
<td>31.09 ± 0.98</td>
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<tr>
<td>Ovary</td>
<td>OVCAR-5</td>
<td>57.71 ± 0.83</td>
<td>48.17 ± 0.78</td>
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<tr>
<td></td>
<td>IGROV-1</td>
<td>64.13 ± 1.55</td>
<td>48.57 ± 0.64</td>
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<tr>
<td>Prostate</td>
<td>PC-3</td>
<td>50.28 ± 1.42</td>
<td>48.07 ± 0.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DU-145</td>
<td>74.59 ± 1.61</td>
<td>48.57 ± 0.62</td>
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</tr>
<tr>
<td>Lung</td>
<td>A-549</td>
<td>48.64 ± 1.15</td>
<td>34.64 ± 1.02</td>
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<tr>
<td></td>
<td>Hop-62</td>
<td>62.19 ± 1.65</td>
<td>43.30 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td>SiHa</td>
<td>71.59 ± 1.57</td>
<td>56.26 ± 0.93</td>
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</tr>
<tr>
<td></td>
<td>Hela</td>
<td>68.10 ± 1.78</td>
<td>40.50 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>Leukemia</td>
<td>HL-60</td>
<td>39.35 ± 0.71</td>
<td>37.10 ± 1.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MOLT-4</td>
<td>69.29 ± 0.91</td>
<td>46.93 ± 0.64</td>
<td></td>
</tr>
</tbody>
</table>

IC50 values of CSL-E and CSL-F of Cannabis sativa leaves against human cancer cell lines. Data are expressed as Mean ± S.E of three similar experiments.

Light microscopy
HL-60 cells treated with CSL-F at 10 and 30 µg/ml for 24 hr exhibited dose-dependent morphological changes such as cell shrinkage, membrane blebbing and formation of apoptotic bodies, which eventually leads to internucleosomal DNA fragmentation and sub G1 accumulation while the nuclei of untreated HL-60 cells appeared round in shape (Fig. 1). Thus, the morphological changes observed in presence of CSL-F indicated induction of apoptosis.

Analysis of apoptosis and necrosis by Annexin V/PI binding
Early events of apoptosis were analyzed using the Apoptosis Detection Kit (APOAF; Sigma Aldrich). To differentiate between apoptosis and necrosis, HL-60 cells (5x10⁶/ml) were exposed to CSL-F (10 and 30 µg/ml) and incubated for 24 hr. All preparations were stained with annexin V-FITC+/PI+ for flow cytometric analysis. Cells were acquired for FSC and SSC and major gated cell population was analyzed for apoptosis and necrosis. The CSL-F at 10 and 30 µg/ml and camptothecin (4 µM) increased the percentage of annexin V positive cells by 15.42, 28.59 and 25.62 respectively whereas annexin V+PI+ cells for the same were 7.50, 14.47 and 4.23% respectively (Fig. 2). The values for the control were
3.32 and 4.51% respectively for annexin V+/PI- and annexin V-PI+ cells. Apoptosis thus appeared to be the primary mode of cell death induced by CSL-F.

**DNA fragmentation by agarose gel electrophoresis**

Analysis of DNA from apoptotic cells by agarose gel electrophoresis is known to produce a characteristic DNA ladder that is widely regarded as a biochemical hallmark of apoptosis. Distinct DNA laddering pattern was observed in CSL-F treated with 10 and 30 µg/ml concentration after 24 hr incubation period. Camptothecin (4 µM) used as a standard also showed a clear DNA ladder while no such ladder was observed in untreated HL-60 cells (Fig. 3).

**Effect of CSL-F on Rhodamine 123 uptake by HL-60 cells**

Early cellular apoptosis is always accompanied by the disruption of mitochondrial membrane, resulting in a rapid collapse in the electrochemical gradient. In the present study, the rhodamine fluorescence was used to determine the mitochondrial membrane potential in HL-60 cells following treatment with CSL-F at the concentration of 10 and 30 µg/ml. From the flow cytometric analysis, as the concentration of CSL-F increased, more cells became susceptible to mitochondrial membrane depolarization (Fig. 5). A remarkable attenuation of \( \Delta \psi_{mt} \) (79.57%) occurred in cells exposed to 30 µg/ml of CSL-F whereas in untreated HL-60 cells, approximately 90% of cells were functionally active with high Rh123 signals.

**CSL-F mediated stimulation of caspases in HL-60 cells**

HL-60 cells treated with CSL-F for 24 hr produced a significant concentration dependent increase in caspase activities. The cells incubated with higher concentration (30 µg/ml) of CSL-F activated caspase-3, -6 and -9 activities by 1.5–2.0 folds (Fig. 6). Caspase-9 activation by CSL-F revealed that it induces apoptosis through intrinsic or mitochondria-dependent pathway.

**PARP cleavage induced by CSL-F**

An additional evidence of caspase-3 activation was the cleavage of PARP as investigated by flow cytometric analysis using a mouse monoclonal antibody. Incubation of HL-60 cells with CSL-F resulted in the formation of cleaved protein.
Mitochondrial dysfunction induced by CSL-F associates with cytochrome c release and Bax translocation

To investigate whether CSL-F treatment induces mitochondrial translocation of Bax concomitant with cytochrome c release to the cytosol, HL-60 cells were treated with CSL-F at 10 and 30 µg/ml for 24 hr. As indicated in the Fig. 8, a concentration-dependent increase in cytochrome c and Bax protein levels was observed in cytosolic and mitochondrial fractions respectively in CSL-F-treated HL-60 cells. These results suggest that CSL-F induces redistribution of the Bax protein to the mitochondria along with cytochrome c release to the cytosol during the progression of apoptosis.

DISCUSSION

Much of the contemporary research in the development of anticancer therapeutics from plants has been focused on investigating the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells. Anticancer drugs inducing apoptosis, having low side effects and target specific cytotoxicity to cancer cells are the drugs of choice. In this regard, the cytotoxic potential of 95% ethanolic extract (CSL-E) and n-hexane fraction (CSL-F) of *C. sativa* leaves was investigated in several human cancer cell lines. The results of the present study

![Graph](image1)

Fig. 5 CSL-F induced concentration-dependent loss of mitochondrial membrane potential (ΔΨ) in HL-60 cells. Numbers indicated in each graph as M2 are percentages of cells with low Rh-123 fluorescence. Data are representative of one of two similar experiments.

![Graph](image2)

Fig. 6 CSL-F induces caspase activation in HL-60 cells. The activities were determined colorimetrically by using BioVision colorimetric caspase assay kits. Data are Mean ± S.E. from three similar experiments. *P < 0.05; **P < 0.01 for treated vs. control.

![Graph](image3)

Fig. 7 CSL-F induces concentration-dependent cleavage of PARP in HL-60 cells. Numbers indicated in each graph as M2 are percentages of cells with cleaved PARP. Data are representative of one of two similar experiments.

![Graph](image4)

Fig. 8 Influence of CSL-F on the expression of cytochrome c and Bax protein involved in the initiation of apoptosis. Data are Mean ± S.E. from three similar experiments. P-values: *P < 0.05; **P < 0.01 for CSL-F treated vs. untreated control cells.

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