Research Article

Litchi chinensis Leaves Cause Cell Death via Caspases on Hepatocellular Carcinoma Cells

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ABSTRACT

Innumerable scientific reports have identified the therapeutic roles of *Litchi chinensis* plant. *Litchi chinensis* fruit pericarp extract and its constituents exhibit anti-cancer activity against human breast cancer and hepatocellular carcinoma *in vitro* and *in vivo*. Anti-inflammatory and anti-leukemic activity of *Litchi chinensis* leaves extract was already reported in our previous work. The present study elaborated the anticancer effects of leaf extract of *Litchi chinensis* (LCLE) on hepatocellular carcinoma cell lines and made efforts to identify the possible mechanism of action involved in the anti-cancer activity. LCLE inhibited the metabolic activities of cells in a concentration-dependent manner. After treatment with the extract, hepatocarcinoma cells, showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies than control cells. LCLE treated cells showed degraded DNA bands and cells were arrested in the G_0/G1 phase of cell cycle. LCLE exerted significant shift in the mitochondrial transmembrane potential activity involving the release of cytochrome c into the cytosol and significant activation of caspase-3.

Keywords: *Litchi chinensis* leaves, Hepatocellular Carcinoma, Apoptosis, Caspase.

INTRODUCTION

Chemoprevention offers a novel approach emphasizing on the prevention or delay of carcinogenesis by means of pharmacological, biological, and nutritional intervention and recently, cancer chemoprevention with strategies using dietary modification, foods and medicinal herbs has been regarded as one of the most striking fields for cancer control[1]. *Litchi chinensis* have been long known for its anticancer, antiviral and antibacterial activities from its ethno-pharmacological history[2]. Study with Litchi leaves has reported for various phytoconstituents such as flavonoids, alkaloids, tannins, terpenoids, saponins and steroid etc. like flavonoids- epicatechin, procyanidin A2 and procyanidin B2 which are responsible for the anti-diabetic, anticancer, antioxidant, free radical scavenging activity and also showed reduced blood sugar level[3]. Potential hepato protection against paracetamol-induced hepatic damage was exhibited by the chloroform and methanol fraction of *Litchi chinensis* which neutralized the biochemical parameters in rats possibly by augmenting endogenous antioxidant defense mechanisms[4]. Our previous study showed the pharmacological studies with the petroleum ether extract of leaves of the Litchi plant was reported to have anti-inflammatory, analgesic and antipyretic activity and induction of apoptosis in human leukemic cell lines U937, K562 & HL-60 by *Litchi chinensis* leaf extract via activation of mitochondria mediated caspase cascades. But no work has been done so far on hepatic carcinoma, to the best of our knowledge. Therefore, in this present study, we have investigated the anticancer effect of leaf extract of *Litchi chinensis* (LCLE) against hepatocellular carcinoma (Huh-7 & HepG2) cell lines and have made efforts to identify the possible mechanism of action involved in the anticancer activity.

MATERIALS AND METHODS

Chemicals

DMEM (Gibco), fetal bovine serum (FBS), Trypsin (Gibco, USA), Penicillin-streptomycin (Biowest, Germany), Gentamycin (Nicholas, India), HEPES, L-glutamine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)], Acridine orange, Ethidium bromide, JC-1 dye, Caspase-3 assay kit, Propidium iodide (Sigma), ethylene diamine tetra acetic acid (EDTA), Agarose (Puregene), Proteinase k (SRL), RNAse, DMSO (dimethylsulphoxide), Chloroform, isoamyl alcohol, Methanol (Merck), and all other chemicals and reagents were of analytical grade and procured locally.

Cell Culture

Huh-7 and HepG2 were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The cells were cultured and routinely maintained in DMEM medium and the medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100µg/ml), gentamycin (100µg/ml) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 inside a CO_2 incubator. Both cell lines were adherent in nature. During sub culturing of cells, the adherent property was diminished by adding 1X trypsin solution in the cell.

Extraction and Preparation of Test Sample

The leaves of *Litchi chinensis* were collected by local supplier from sites around Kolkata, India, during June

2006. The plant was identified by Indian Botanical Garden, Howrah, India, where a voucher specimen (SR-002) has been kept. One kg of *Litchi chinensis* leaves were sun-dried, ground and soaked in 3 liter of 50% aqueous methanol for one week at room temperature (28-34°C) with occasional shaking. The supernatant was filtered and to the residue 1 liter of 50% aqueous methanol was added and kept for another one week. The methanol portion in the whole supernatant was evaporated using a rotary evaporator and then it was lyophilized to remove the water. The solid dark brown residue (35g) thus obtained was designated as '*Litchi chinensis* leaf extract (LCLE)' and was kept at 4°C. From 1 mg/ml stock solution of LCLE in phosphate buffered saline (PBS), different suitable concentrations like 25, 50, 100 and 200 µg/ml were used for different *in vitro* experiments.

Cytotoxicity Analysis

Huh-7 and HepG2 cells ($1x10^5$) were seeded in 96-well sterile plates and incubated in CO_2 incubator for 24h. Cells were then treated with freshly prepared LCLE at different concentrations (25, 50,100, 200 µg/ml) and time intervals in a humidified atmosphere containing 5% CO_2. Cell growth inhibition studies were performed by MTT assay and the absorbance of this colored solution was quantified by measuring at certain wavelength-570 nm by micro-plate manager (Reader type: Model 680 XR Bio-Rad Laboratories Inc.). The IC_{50} values for both the cell lines were determined at 24h.[5]

Morphological Analysis

Huh-7 and HepG2 cells ($1x10^6$) treated with IC_{50} of LCLE for 24h were observed using both fluorescence and confocal microscope for morphological changes. The untreated control cells and the LCLE treated cells were harvested separately, washed with PBS twice and then stained with acridine orange (100µg/ml) and ethidium bromide (100µg/ml) (1:1) and the cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis. For confocal microscopic study both the cells were stained with 10µg/ml of propidium iodide separately for 5 min. After mounting on slides the cells were observed to see the differences in nuclear morphology between the untreated and the LCLE treated hepatoma cells under confocal laser scanning microscope (Olympus, Fluoview FV10i) at 60X. Images for propidium iodide were acquired from argon/krypton laser and UV laser line using 590 nm long pass filter for propidium iodide and 450 nm band pass filter for UV images.

DNA Fragmentation Analysis

Huh-7 and HepG2 cells were treated with IC_{50} of LCLE for 24h. The cells were harvested and washed twice with PBS. The cells were resuspended in 500µl of lysis buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 0.5% SDS), 100µg/ml of proteinase K was added and incubation was done at 55°C for 1h and 37°C overnight respectively. DNA extraction was done by following the general phenol-chloroform extraction procedure[7] and kept at -20°C overnight. After centrifugation, DNA precipitates were washed with 70% chilled ethanol, then dried and evaporated at room temperature and dissolved in TE buffer (pH 8.0) at 4°C overnight. To detect the DNA fragments, the isolated DNA samples were electrophoresed overnight at 20V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator (GENEI, Bangalore Genei Pvt. Ltd.).

Mitochondrial Membrane Potential (Δψm) Analysis

Huh-7 and HepG2 ($1x10^6$) cells were treated with LCLE with IC_{50} dose and control for 24 hours to assay the mitochondrial membrane potential activity of cell in a flow cytometer. Cells were washed with PBS, pelleted down and eventually stained with JC-1 stain. The sample was incubated at 37°C for 15 min. Shift in the mitochondrial membrane potential was determined by FACS (Becton Dickinson FACS Fortessa 4 leaser cytometer), Florence detector equipped with 520 nm argon laser light source and 623 nm band pass filter (linear scale) with the help of BD FACS Diva software (Becton Dickinson).

Cell Cycle Analysis

In order to study the stage of cell cycle arrest in flow cytometry, Huh-7 and HepG2 cells ($1x10^6$) were treated with IC_{50} of LCLE for 18h. Cells were washed with PBS, fixed with cold methanol by adding methanol drop-wise and kept at -20°C for 3 min. They were then resuspended in cold PBS and kept at 4°C for 90 min. Cells were pelleted down, then dissolved in cold PBS, treated with RNase A for 30 min at 37°C and stained with propidium iodide (20µl from 50µg/ml) and kept in dark for 15min. Cell cycle phase distribution of nuclear DNA was determined on BD FACS Diva software (Becton Dickinson FACS).

Caspase-3 Assay

The assay was performed using a Caspase-3 Assay kit, Colorimetric (Sigma) according to the manufacturer's protocol. HepG2 cells ($1x10^6$) were treated with IC_{50} dose of LCLE for 24h. The untreated control and the treated cells were pelleted down by centrifugation at 600 xg for 5min at 4°C. Supernatants were removed and the cell pellets were washed with 1ml of PBS. The cells were again centrifuged and the supernatants were removed completely. The cell pellets were suspended in 100µl of 1X lysis buffer (50mM HEPES, pH 7.4, 5mM CHAPS, 5mM DTT) and incubated on ice for 20min. The lysed cells were centrifuged at 20,000 xg for 15min at 4°C and the supernatants (cell lysates) were analyzed for the caspases-3 activity according to the manufacturer's protocol. Cell lysates were incubated with 2mM Caspase-3 substrate (Ac-DEVD-pNA) in 1X assay buffer (20mM HEPES, pH 7.4, 2mM EDTA, 0.1% CHAPS, 5mM DTT) for 90min at 37°C. The absorbance was read at 405nm and the results were calculated using a p-nitroaniline calibration curve.

Statistical Analysis

Percentage of cell growth inhibition was calculated by the following formula:

% Cell Inhibition = 10 x (O.D of Control –O.D of treated /O.D of Control), where O. D= Optical Density.

Percentage of cell viability was calculated as follows:

Viable Cells (%) = (Total number viable cells per ml/Total number of cells per 1ml) X 100.

RESULTS

Cytoxicity Analysis

Cytotoxicity assays were carried out on hepatoma cell lines such as Huh-7 and HepG2 with LCLE by MTT assay. The results displayed in Fig. 1, suggest that the LCLE significantly inhibited the growth of metabolically active cells in a time and concentration-dependent manner. These observations provided proof for cytotoxic nature of LCLE in both the hepatoma cells. The IC_{50} value of LCLE was, for Huh-7 **72.66µg** and for HepG2 **50µg.**

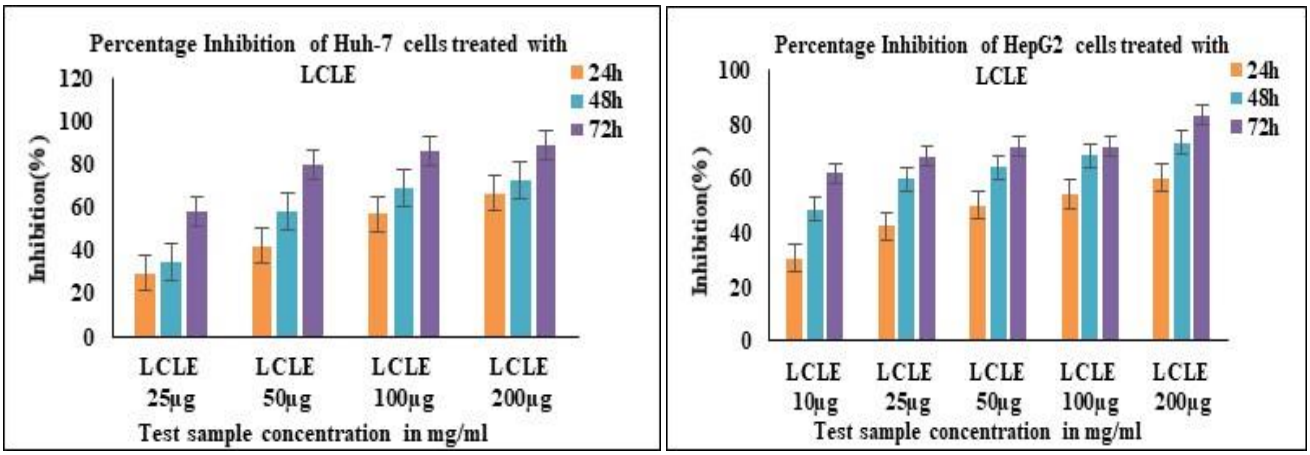

Figure 1: Cytotoxicity of LCLE on Huh-7 and HepG2 cell lines by MTT assay for 24, 48 an72h.

Data showing % inhibition of LCLE treated cells compared to control cells are represented as mean ± S.E. (*n* =4).

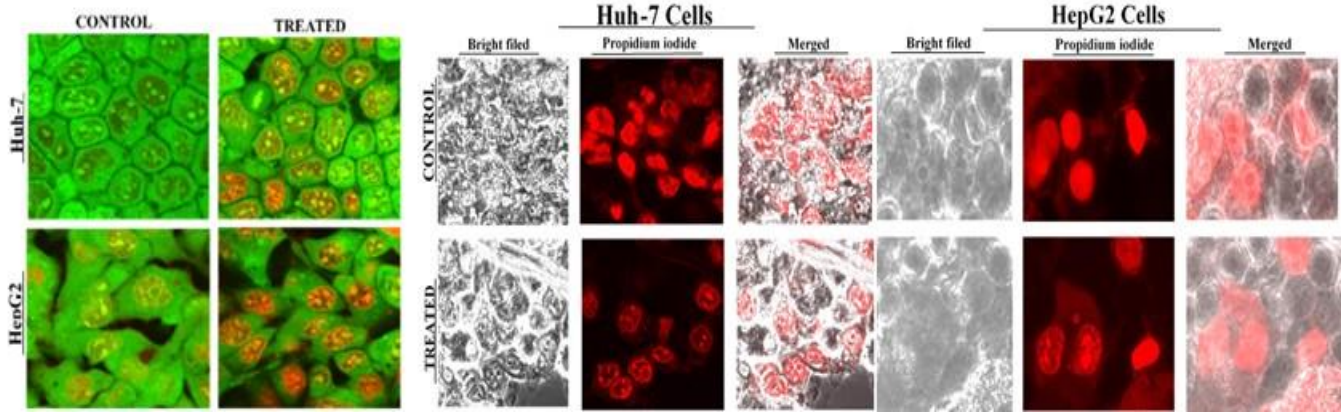

Figure 2: Fluorescence and confocal microscopic images of untreated and LCLE treated Huh-7 and HepG2 cells using acridine orange and ethidium bromide. Untreated cells showed bright green fluorescence with intact membrane structure, no nuclear damage where as LCLE treated both the cells showed membrane blebbing and chromatin disintegration (60 x magnification). In Confocal microscopic images of both the cells confirmed the distinct nuclear fragmentation after 24 hours of treatment with LCLE.

Morphological Analysis

Observations revealed that both the microscopic images of Huh-7 and HepG2 cells indicated the fact that treatment with LCLE brought about apoptotic changes in the cells like condensation of chromatin and nuclear fragmentation the sign of apoptosis. (Figure 2).

DNA Fragmentation Analysis

The gel pattern of the DNA samples isolated from control Huh-7 and HepG2 cells showed intact DNA bands, whereas the gel pattern of the DNA samples isolated from LCLE treated both the cells showed degraded DNA bands in the form of ladders. So, the observations confirmed that the treatment with LCLE caused apoptosis in the cells. (Figure 3).

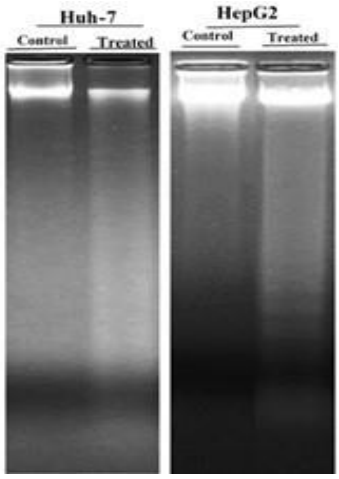

Figure 3: The gel pattern shows DNA fragmentation. DNA isolated from Huh-7 and HepG2 cell. The lane 1 represent Huh-7 and HepG2 control cells which shows intact DNA whereas Lane 2 represent LCLE IC_{50} dose treated cell in which DNA fragments are clearly visible.

Mitochondrial Membrane Potential (Δψm) Assay

Mitochondrial dysfunction is an essential target for induction of apoptosis. The hepatocellular carcinoma cell lines (Huh-7 and HepG2) when treated with LCLE (IC_{50} dose), there was a loss of Mitochondrial Membrane Potential (Δψm).

The JC-1 stain cannot accumulate in the mitochondria of the apoptotic cells, as the mitochondrial membrane potential collapses, hence showing green fluorescence (P4) denotes apoptotic cells and red fluorescence (P3) denoting healthy cells where JC-1 stain accumulates. Depolarization in mitochondrial membrane potential was observed by staining untreated and treated cell by JC-1 dye. The depolarization led to a transmembrane shift from red to green fluorescence leading to release of cytochrome c. A significant transmembrane shift of **8.1** to **34.9** (Huh-7) and **9.1** to **29.9** (HepG2) was observed when Huh-7 and HepG2 cells were treated with the IC_{50} value of LCLE for 24 hours.

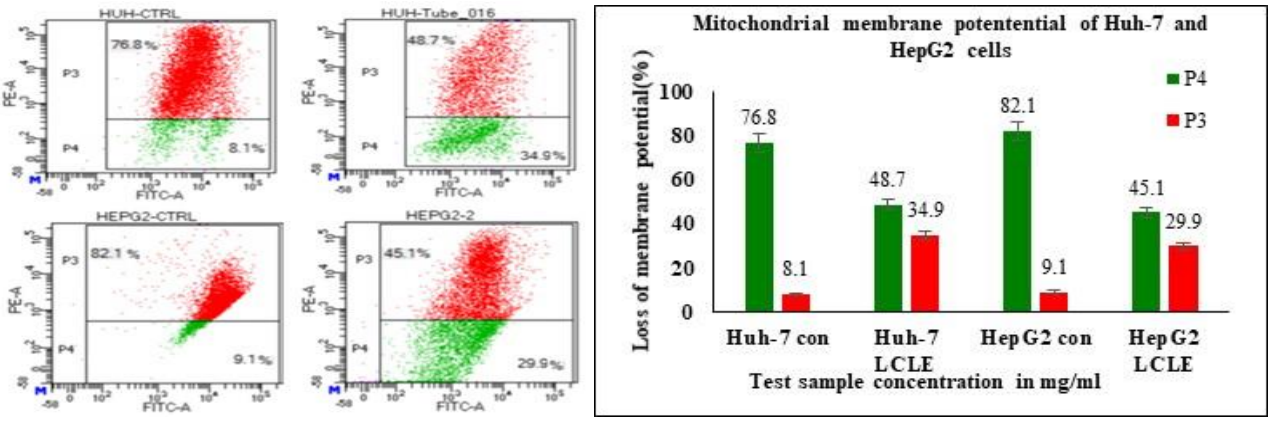

Figure 4: Mitochondrial membrane potential activity of LCLE on Huh-7 and HepG2 cells after 24hrs of treatment. Histogram showed fold increase of mitochondrial membrane potential(Δψm) in both the cells when treated with IC_{50} dose of LCLE.

Cell Cycle Analysis

Flow cytometry analysis showed that after 24hrs of treatment of Huh-7 and HepG2 with LCLE at IC_{50} dose, sub-G1 peak was changed. The DNA content increased in LCLE treated cell **58.5% against 37.8% in Huh-7** and **60.2% against 45.6% in HepG2** cells. These observations revealed that LCLE inhibited the growth of Huh 7 and HepG2 cells by arresting the cell populations in the sub-G_0/G1 phase of the cell cycle.

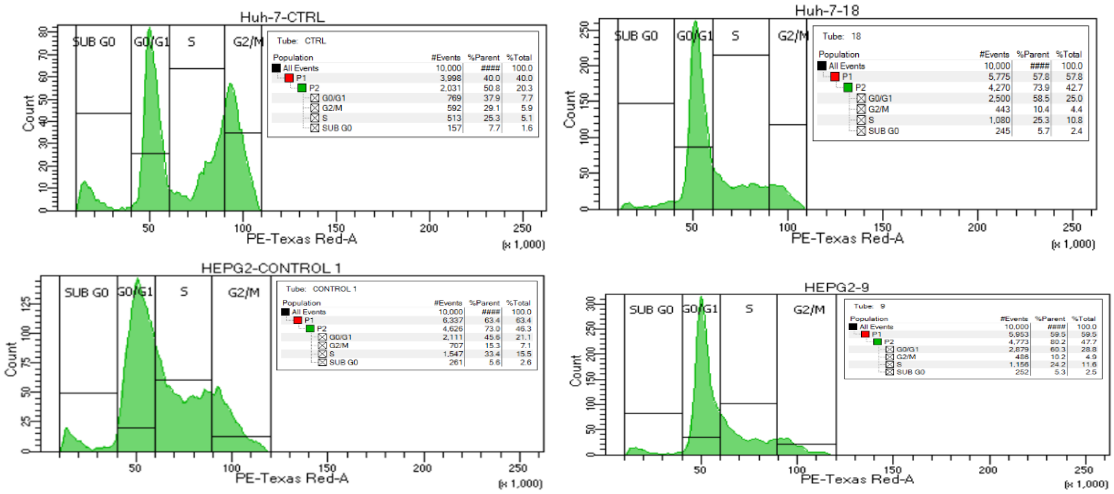

Figure 5: Flow cytometry analysis of cell cycle phase distributon in control and treated cells of Huh-7 and HepG2 respectively after 18 hrs of treatment at IC_{50} dose of LCLE.

Caspase-3 Assay

Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspase-3 activation was clearly observed in LCLE treated HepG2 cells when compared with that of the untreated control cells. The experiments revealed that there was 3.1-fold increase in the caspase-3 activity in the cytosolic extract of LCLE (IC_{50}) treated HepG2 cells compared with that of the untreated control cells.

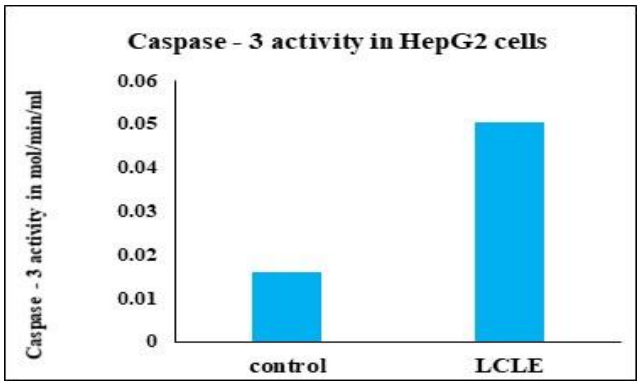

Figure 6: Histogram showing the fold increase in Caspase-3 activity on HepG2 cells treated with LCLE (IC_{50} dose).

DISCUSSION

The purpose of the present study was to investigate the anti-cancer activity of aqueous methanol extract of *Litchi chinensis* leaves (LCLE) against human hepatocellular carcinoma cell lines (Huh-7 and HepG2). The cytotoxic activities of LCLE were supported by the observations in MTT assays. Inhibition in growth of metabolic activities of both the hepatoma cells by LCLE was evidenced in a concentration-dependent manner. Apoptosis activity of LCLE was examined by different morphological studies like fluorescence and confocal microscopic. The process of apoptosis which is characterized by several morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies was observed. Fluorescence microscopic images clearly showed nuclear disintegration of LCLE treated both the hepatocellular carcinoma cells compared with that of the untreated control cells when stained with acridine orange and ethidium bromide. The untreated control cells showed bright green fluorescence as the live cells with intact membrane excluded ethidium bromide and only acridine orange could enter into them. On the contrary LCLE treated cells showed more intense orange-red fluorescence and reduced green fluorescence. The observations indicated that the treatment with LCLE was inducing apoptosis in the hepatocellular carcinoma cells. Apoptosis activity of LCLE was further evidenced from the confocal microscopic images of the treated hepatocellular carcinoma cells when compared with that of the untreated control cells. After LCLE treatment, Huh-7 and HepG2 cells showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei.

Further evidence in support of the anti-cancer activity of LCLE was obtained from the gel patterns of agarose gel electrophoresis. Cells treated with LCLE showed degraded DNA bands in the form of ladders, a typical indication of apoptosis, whereas the untreated control cells showed intact DNA bands when observed in UV transilluminator[7]. The mitochondrial membrane potential assay by JC-1 dye showed increase in apoptotic cells after treatment with LCLE implying the fact that apoptosis was triggered by the treatment with LCLE in Huh-7 and HepG2 cells. Cell cycle analysis revealed that treatment with LCLE arrested the Huh-7 and HepG2 cell populations in the G_0/G1 phase of cell cycle.[8]

There are two major apoptotic pathways known to date, initiated by either mitochondrion (the 'intrinsic' pathway) or the cell surface receptors (the 'extrinsic' pathway).[9] Mitochondria-mediated apoptosis occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p53) and oncogenes (such as c-Myc), DNA damage, chemotherapeutic agents, serum starvation, and ultraviolet radiation[10]. The experiment with caspase-3 assay revealed 3.1fold increase in the caspase-3 activity in the HepG2 cells treated with cytosolic extract of LCLE (IC_{50}) compared with that of untreated control HepG2 cells. These observations in the present work suggested that treatment with LCLE, induced apoptosis via mitochondria mediated intrinsic pathway in hepatoma cell populations. From all the above data, it can be concluded that LCLE possesses anticancer effect against hepatocellular carcinoma cells.

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