Chelidonine isolated from ethanolic extract of *Chelidonium majus* promotes apoptosis in HeLa cells through p38-p53 and PI3K/AKT signalling pathways

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**OBJECTIVE:** To evaluate the role of chelidonine isolated from ethanolic extract of *Chelidonium majus* in inducing apoptosis in HeLa cells and to assess the main signalling pathways involved.

**METHODS:** Cells were initially treated with different concentrations of chelidonine for 48 h and the median lethal dose (LD₅₀) value was selected by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Morphological analysis of nuclear condensation and DNA damage and fragmentation were measured by 4′,6-diamidino-2-phenylindole staining and comet assay. Further, reactive oxygen species (ROS) generation, cell cycle arrest and change in mitochondrial membrane potential were also examined and analyzed by flow cytometry. Evaluation of interaction of drug with CT DNA was investigated by circular dichroism (CD) spectral analysis to find any possible drug-DNA interaction. The mRNA and protein expressions of major signal proteins like p38, p53, protein kinase B (AKT), phosphatidylinositol 3-kinases (PI3K), Janus kinase 3 (JAK3), signal transducer and activator of transcription 3 (STAT3) and E6 and E7 oncoproteins as well as the pro-apoptotic genes and antiapoptotic genes were also estimated by reverse transcriptase-polymerase chain reaction and Western blotting.

**RESULTS:** Based on LD₅₀ value (30 μg/mL) of chelidonine, three doses were selected, namely, 22.5 μg/mL (D1), 30.0 μg/mL (D2) and 37.5 μg/mL (D3). Results showed that chelidonine inhibited proliferation and induced apoptosis in HeLa cells through generation of ROS, cell cycle arrest at sub-G₀ and G₀/G₁ stage, change in mitochondrial membrane potential...
and fragmentation of DNA. Results of CD spectra showed effective interaction between chelidonine and calf thymus DNA. Studies of signalling pathway revealed that chelidonine could efficiently induce apoptosis through up-regulation of expressions of p38, p53 and other pro-apoptotic genes and down-regulation of expressions of AKT, PI3K, JAK3, STAT3, E6, E7 and other antiapoptotic genes.

**CONCLUSION:** Chelidonine isolated from *Chelidonium majus* efficiently induced apoptosis in HeLa cells through possible alteration of p38-p53 and AKT/PI3 kinase signalling pathways.

**KEYWORDS:** chelidonine; *Chelidonium majus*; antineoplastic agents, phytogetic; signal transducing; apoptosis; HeLa cells

*Chelidonium majus* is commonly known as greater celandine. It belongs to the family Papaveraceae and is widely distributed in Europe and Western Asia\(^1\). The crude extracts of various parts such as the root, shoot and leaves have been reported to have several isooquinoline alkaloids, such as sanguinarine, chelidonine, chelerythrine, berberine and coptisine. Both crude extracts of *C. majus* and purified compounds derived from it have been reported to exhibit a wide variety of biological activities like antiviral anti-inflammatory, antimicrobial, immune-modulatory, antitumoral, choleretic, hepato-protective and analgesic which are in accordance with the traditional uses\(^2\).

Earlier studies suggest that, the plant extract of *C. majus* and its derivatives have potentials of being successfully used as a therapeutic agent against lung\(^3\), liver\(^4\), pancreatic\(^5\), prostate\(^6\) and breast\(^7\) cancers; they also can act as an anti-mitotic agent\(^8\).

Human papilloma virus (HPV)-induced mutation in cervix may lead the cells to an uncontrollable proliferation state called neoplasia\(^9\). Therefore the search is relentlessly on to find out effective chemotherapeutic agents against the cervical carcinoma that can reduce the prevalence and mortality of this disease.

Since cancer cells are immortal and proliferate indefinitely in culture, the anticancer drugs should have the potential of induction of apoptotic pathway and reduction in cell proliferation\(^10\). These two processes can be simultaneously controlled by up- and down-regulation of signal proteins, one of them being p38 mitogen-activated protein kinase (MAPK), which regulates the cell cycle and controls the expression of different transcription factors like p53, protein kinase B (AKT) and Janus kinase (JAK), which further have specific regulatory role in cell proliferation and apoptosis.

Reactive oxygen species (ROS) is one major component which makes the cell apoptosis by changing different events either by force stoppage in cell cycle at sub-G\(_1\) or G\(_0\)/G\(_1\) stage or by making the mitochondrial outer membrane permeable. Thus the assessments of these three factors are major critical decision-making junction points for initiation of apoptosis and reduction of cell growth. The evaluation of interaction between the drug and cellular DNA is a major molecular event to elucidate working principle of the drug at the molecular level. The DNA-binding capacity of the drug can provide us with a clue as to if the drug is interacting directly at the DNA level, and this could, in turn, be reflected in relevant change in the mRNA level expression.

In the present study, the hypothesis to be tested is whether the isolated chelidonine from ethanolic extract of *C. majus* (EECM) has therapeutic effects against HeLa human cervix cancer cell line. Here our main point of focus will be to know
whether the drug acts mainly through modulations in the signal pathways involving p38 network by inhibition of phosphatidyl inositol 3-kinase (PI3K), AKT, signal transducer and activator of transcription 3 (STAT3)-JAK3 and by up-regulation of p53 network.

1 Materials and methods

1.1 Chemicals and reagents All chemicals used were of analytical grade. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin-neomycin (PSN) antibiotic, trypsin and ethylene-diamine-tetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Bioscience (USA). All the antibodies and annexin V-fluorescein isothiocyanate (FITC) used were obtained from Santa Cruz Biotechnology (USA). Propidium iodide (PI), acrydine orange (AO), 4’-6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), ethidium bromide (EB), rhodamine 123, 2’,7’-dichlorodihydrofluorescein diacetate (DCHF-DA) and all other chemicals were purchased from Sigma Chemical Co. (USA). All other reagents were used of high-performance liquid chromatography grade. EECP was obtained from the Bioron Laboratory (France).

1.2 Chromatographic separation and characterization of EECP The solvent (ethanol) from EECP was evaporated to a thick residue and mixed with silica gel (60 to 120 mesh) and air dried. The mixture was then used for separation by standard column chromatography method with different volumetric ratios of chloroform and methanol (silica gel 60 to 120) and then further purified by preparative thin-layer chromatography, and the collected fractions were analyzed by mass spectroscopy and Fourier transform infrared spectroscopy (FTIR) [11].

1.3 Cell culture HeLa cell line was obtained from the National Centre for Cell Science, Pune, India. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic (PSN) and maintained at 37 °C with 5% CO₂ in a humidified incubator. Cells were harvested with 0.025% trypsin and 0.52 mmol/L EDTA in phosphate buffer saline (PBS) and plated at required cell numbers and allowed to adhere for 24 h before treatment.

1.4 MTT assay The HeLa cells were dispensed in 96-well flat bottom microtiter plates at a density of 1×10⁴ cells per well. After 24 h of incubation, they were treated with various concentrations of isolated chelidonine and incubated for 48 h to determine the concentrations of drugs at which, the percentage of cell death was nearly median lethal dose (LD₅₀). The positive control received no drugs but only the alcohol. After the incubation, 10 μL of MTT solution (5 mg/mL) was added to each well. The intracellular formazan crystals formed were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Multiscan EX, Thermo Electron Corporation, USA). The relative percentage of viability was calculated as follows: relative percentage viability = (optical density (OD) of the drug-treated sample/OD of the control sample) × 100%.

1.5 Treatment types We selected three different doses of isolated chelidonine depending on the LD₅₀ data for further experiments. They were 22.5 μg/mL (D1), 30.0 μg/mL (D2), and 37.5 μg/mL (D3). DMEM was used to dissolve the drug (isolated chelidonine). Cells receiving only medium but not drug serve as the control for comparing the experimental data.

1.6 Lactate dehydrogenase cytotoxicity assay Lactate dehydrogenase (LDH) activity was assessed using a standardized kinetic determination kit (Enzopak, Recon, India). LDH activity was measured in both floating dead cells and adherent viable cells for control and drug-treated cells. The floating cells were collected from culture medium by centrifugation (500 × g) at 4 °C for 2 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHₐ) [18]. The LDH released in the culture supernatant (designated as extracellular LDH, LDHₑ) was used as an index of necrotic death, and the LDH present in the adherent viable cells was designated as intracellular LDH (LDHᵢ). The percentages of apoptotic and necrotic cell deaths were calculated as follows:

Apoptosis percentage = [LDHₑ/(LDHₑ + LDHᵢ + LDHₐ)] × 100%

Necrotic percentage = [LDHᵢ/(LDHₑ + LDHᵢ + LDHₐ)] × 100%

1.7 Observation of morphological changes Cells plated in six-well culture plates (1×10⁴ cells per well) were received either with one of the drug treatments (D1, D2 or D3), or with the respective positive control. After 48 h, the cells were observed under an inverted phase-contrast microscope (Axioskop plus 2, Zeiss, Germany) and the photographs were taken.

1.8 Fluorescence microscopy Cells treated for 48 h were stained separately with DAPI (10 μg/mL), or AO-EB. Then the cells were analyzed under a fluorescence microscope (Axioskop plus 2, Zeiss, Germany) and representative photographs were taken for further quantitative and qualitative analyses.

1.9 Comet assay After treatment and incubation, the cells were trypsinized and washed in ice-cold PBS. The cell suspension was mixed with an equal amount of 0.75% low-melting agarose and kept at 37 °C. Immediately after mixing, 100 μL of the suspension was pipetted onto a microscope slide pre-coated with high-electroendosmosis (EEO) agarose, then covered with cover slip and placed
on a glass tray on ice. Then the slides were immersed in cold lysis solution (2.5 mol/L sodium chloride (NaCl), 100 mmol/L EDTA, 10 mmol/L Tris, pH 10), with freshly added 1% Triton X-100 and 2% dimethyl sulfoxide followed by incubation at 4 °C for at least 1 h. The electrophoresis in weak alkali (0.03 mol/L sodium hydroxide, 1 mmol/L EDTA, pH 12) at 1 V/cm and 30 mA for 15 min was preceded by a 20 min immersion of the slides in electrophoresis buffer to promote chromatin unwinding. After electrophoresis the slides were neutralized in 0.05 mol/L Tris buffer. Then DNA was stained with EB (50 μg/mL) for 10 min, washed in distilled water and examined under a fluorescence microscope (Lycia, USA)\(^{10}\).

1.10 DNA fragmentation assay  The cells were grown to 70% confluence and treated with D1, D2 and D3 for 48 h. DNA of the control and treated (with indicated concentrations) cells was isolated by standardized phenol-chloroform method and DNA gel electrophoresis was performed in 1% agarose gel. The bands were visualized under an ultraviolet transilluminator followed by digital photography\(^{20}\).

1.11 Analysis of interaction of the isolated chelidonine with calf thymus DNA by circular dichroism spectra  Circular dichroism (CD) spectra showed changes in the structure of DNA, which were monitored in the region of 200 to 450 nm, using 1 cm path length cells (Jasco spectropolarimeter; model PC controlled J-815; Jasco International Co. Ltd.; equipped with a temperature controller and thermal programmer PFO 425L/15). The DNA and the drug concentrations in the experimental sets were 1.0 mmol/L and 30 μg/mL respectively. Experiments were conducted at 25 °C\(^{21}\).

1.12 Flow cytometric analysis  
1.12.1 Detection of apoptosis  HeLa cells of 1 × 10\(^6\) per sample were taken into small centrifuge tubes. Cells were spun at 1,200 × g for 5 min and pellets were washed with 500 μL binding buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; 150 mmol/L NaCl; 5 mmol/L potassium chloride; 1 mmol/L magnesium chloride and 1.8 mmol/L calcium chloride). Then cells were harvested at 1 200 × g for 5 min and cell pellet was resuspended in 80 μL binding buffer; 10 μL annexin V-FITC and 10 μL of PI labelling (10 μg/mL) solutions were added and the cell suspension was incubated for 15 min at room temperature in dark. Then the solution was analyzed by fluorescence-activated cell sorter (FACS, BD Bioscience)\(^{22}\).

1.12.2 Analysis of cell cycle  The cells with hypoploid DNA were determined by cell cycle studies as described earlier. After treatment, HeLa cells were suspended in PBS and fixed by addition of 70% ice-cold methanol. The fixed cells were harvested, washed with PBS and RNase (1 g/L) was added to the samples and resuspended in PI staining (10 μg/mL). The PI fluorescence was measured through an FL-2 filter (585 nm) and 10 000 events were acquired. FACS data were analyzed using Cell Quest Pro software. Histogram display of DNA contents (X-axis, PI fluorescence) versus counts (Y-axis) was provided. Representative data from three independent experiments were furnished.

1.12.3 Quantification of intracellular ROS  Estimation of intracellular ROS was done using cell permeable fluorescent-probe H\(_2\)DCFH-DA, a non-fluorescent compound, which is converted into highly fluorescent dichlorofluorescein (DCF) by cellular peroxides. Cells, after incubation with D1, D2 and D3 for desired time, were loaded with 10 μmol/L H\(_2\)DCFH-DA. Following incubation at 37 °C for 30 min in dark, cells were washed with PBS and fluorescence was monitored with a fluorescence microscope (Axioscope plus 2, Zeiss). The level of intracellular ROS was also measured by FACS with an excitation wavelength of 480 nm and an emission wavelength of 530 nm using the same fluorescence probe.

1.12.4 Measurement of mitochondrial membrane potential (ΔΨ\(_m\))  The changes in mitochondrial membrane potential (MMP) of cells in response to drug (D1, D2, and D3) treatment were determined using a fluorescent probe, rhodamine 123. Loss in MMP was verified through FACS at single cell level. After treatment, cells were washed in ice-cold PBS before incubation with rhodamine 123 (5 mmol/L) in dark for 15 min at room temperature. The probe was excited at 488 nm and emission was measured through a 530 nm band-pass filter. Logarithmic amplification was used to detect the fluorescence of the probe.

1.13 Western blot analysis  Cell lysate was prepared as protocol followed by Yang et al\(^{23}\). The cell lysate was then centrifuged at 500 × g for 45 min at 4 °C. The supernatant was collected, and the protein concentration was measured using bovine serum albumin (BSA) as a standard. Aliquots of supernatant (30 μg protein) were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a micropore membrane (Taurus-scientific, USA). The membranes were rinsed three times with Tris-buffered saline (TBS) and blocked for 3 × 30 min with 3% BSA/ TBS/0.1% Tween-20. The membranes were then incubated overnight at 4 °C with monoclonal antibodies specific for each protein (p53, p13K, p53, caspase 3, E6 and E7). Then, the membranes were further incubated for 2 h with specific secondary antibody diluted in TBS. Bound antibodies were developed by 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium and the band intensity was measured using ImageJ software (ImageJ software is a public domain initially produced by Richard Stallman at NIH, USA). Glyceraldehyde-3-phosphate dehydrogenase was used as the house-keeping gene.
1.14 Indirect ELISA Nuclear extract was prepared as followed by Bhattacharyya et al.\textsuperscript{221}. ELISA was carried out in nuclear extract to study the expressions of p38, p53, PI3K, AKT, JAK3, STAT3, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (BAD), B-cell lymphoma-extra large (Bcl-XL), cytochrome c, caspase 9, caspase 3, and apoptotic peptidase activating factor-1 (APAF-1) in control and drug-treated cells quantitatively. Wells were coated with 70 μg of proteins in 100 μL of 0.05 mol/L carbonate buffer (pH 9.6) and kept overnight at 4°C in a moist chamber. The plate was flicked to remove the unbound antigen solution and the wells were blocked with 1% BSA in 0.1 mol/L PBS (pH 7.2) for 1 h at 4°C. The wells were washed thrice with 200 μL of PBS containing 0.05% (v/v) Tween-20. Wells were then incubated with primary antibody (1:500), diluted in PBS (containing 0.05% Tween-20 and 1% BSA) and kept overnight at 37°C. Plate was again washed and incubated with specific secondary antibodies (1:1000) for 2 h at 37°C. Wells were washed further three times as described above and colour was developed by addition of substrate buffer and absorbance at 405 nm was measured by an ELISA reader (Multiscan Ex, Electron Corporation, USA).

1.15 Reverse transcription-polymerase chain reaction analysis Total RNA was extracted from the drug-treated and control HeLa cells, using TRIzol reagent according to the manufacturer’s instructions to estimate the expression of p53, PI3K, AKT, Bcl-2, Bax, caspase-3, poly adenosine diphosphate ribose polymerase-1 (PARP-1), and inhibitor of caspase-activated DNase (ICAD) at their mRNA level. RNA was diluted to 2 μg/mL with water pretreated with diethylpyrocarbonate (DEPC), containing 1 U/μL RNase inhibitor. The ingredients were placed into a tube (1 μL RNA, 0.1 μL oligo (dT) 18, 1 μL reverse transcriptase (RT), 4 μL 10 mmol/L deoxynucleoside triphosphates (dNTP), 4 μL 5 × buffer, total volume made up by sterilized MilliQ water up to 20 μL). The mixture was incubated at 37°C for 60 min. After reverse transcription, the sample was heated at 95°C for 5 min to denote the RT, and then stored at −20°C for polymerase chain reaction (PCR). The synthetic oligonucleotide primers (Table 1) used for RT-PCR were procured from Chromas Biotech, India. cDNA was used as the template for PCR amplification. After 5 min at 94°C for initial denaturation, amplification was performed for all samples under the following conditions: 94°C for 30 s, 52 to 61°C for 30 s (according to the Tm value of the primers) and 72°C for 30 s for 35 cycles, with a final extension at 72°C for 10 min. Following PCR, 5 μL sample aliquots were subjected to electrophoresis on 1% (weight/volume) agarose (medium EEO) gel for 20 to 30 min and then stained with EB and documented\textsuperscript{211}. Densitometric analysis was performed using the imaged software.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
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| Caspase 3   | Forward: 5′-AGGGCTATTTATGGGACA-3′  
               | Reverse: 5′-TACAGGTGCATGTTTCTTGT-3′ |
| PARP-1      | Forward: 5′-ATTCCCACATCTTCTTCTTACCA-3′  
               | Reverse: 5′-GGGCAATAGTCATACAGCAT-3′ |
| PI3K        | Forward: 5′-TTAACGGGAAGGGCAAGGA-3′  
               | Reverse: 5′-CATGCTCTCTGCTGTGCTGAT-3′ |
| AKT         | Forward: 5′-CCCTGACTACCTGCACACTGCTGAA-3′  
               | Reverse: 5′-TTGCTTTCGAGGCTCTGCAAAAGG-3′ |
| Bax         | Forward: 5′-AGTAACTAGGGAGTGCGAGG-3′  
               | Reverse: 5′-ATGGTTTCTGATCAGTTCCGC-3′ |
| Bcl-2       | Forward: 5′-GTACTCCGATCGTGAAGAGA-3′  
               | Reverse: 5′-CCCTGACAGATCGAAGGAC-3′ |
| ICAD        | Forward: 5′-ATGGTCAACGCAGAGCAGAC-3′  
               | Reverse: 5′-GCTGTTTCAATAATTTAAAATGCA-3′ |
| p53         | Forward: 5′-GAAGTTTGGTATCCCGATATCCTG-3′  
               | Reverse: 5′-GTCTTCTGAGTTAGTGTGTTA-3′ |
| GAPDH       | Forward: 5′-CCCATTAAACACATATGGGAGG-3′  
               | Reverse: 5′-CTGTCCCAATGTCGAAATT-3′ |

RT-PCR: reverse transcription-polymerase chain reaction; PARP-1: poly adenosine diphosphate ribose polymerase-1; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase B; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; ICAD: inhibitor of caspase-activated DNase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

1.16 Blinding The observers were blinded during observation as to whether they were observing the control or drug-treated materials.

1.17 Statistical analysis Data were presented as mean ± standard error of mean. Statistical analysis was performed by Student’s paired t-test, one-way analysis of variance and post-hoc analysis using SPSS 10.0 software. P < 0.05 was considered significant.

2 Results

2.1 Characterization of chromatographic fraction The fractions of EECD obtained from column chromatography were subjected to mass spectroscopy and FTIR for characterization. We tested each fraction for its possible anticancer activity by MTT assay. Then we selected the specific fraction which had more palpable anticancer potentials. This fraction had been isolated at the ratio of solvent chloroform : methanol of 6 : 3 in volume and the characterized data of that fraction are as follows.

FTIR data peak at 2 963, 2 919, and 2 850/cm represents C-H (single bond), 1 261/cm represents C-O (single bond), 1 464/cm for C = N group (data not shown)\textsuperscript{221}. The mass spectroscopy data showed the molecular weight of the desired compound to be 353.22 (MS: m/z = 353.22 (M-H)+ ), and the overall spectral data of this compound revealed it to be a chelidonine (C19H19NO2)-rich compound (Figure 1).
2.2 Selection of doses To check the apoptotic capability of isolated chelidone on HeLa cells, we performed the MTT assay for 48 h of different doses of the drugs, which in turn helped us to determine the LD₅₀ value (30 μg/mL). See Figure 2. The control did not show any cytotoxicity and apoptotic effects. The cytotoxicity of D₁, D₂, and D₃ was also checked for peripheral blood mononuclear cells, which also showed a minimal effect (Figure 3).

The ratios of LDH release from viable cells, floating dead cells, and the culture medium were compared and considered for assigning either "apoptotic" or "necrotic" nature. There was a significant increase in the ratio of apoptotic cells for D₁, D₂, and D₃, but those of necrotic cells were still negligible in the presence of one of the three doses of D₁, D₂ and D₃ (Table 2).

![Figure 1](image1.png)

**Figure 1** Mass spectroscopic data of isolated chelidone and the chemical structure.

![Figure 2](image2.png)

**Figure 2** Effects of different concentrations of chelidone on HeLa cells incubated for 48 h tested by MTT assay. Data were expressed as mean±standard error of mean. Values were measured in three independent experiments done in triplicate. *P<0.01, vs control group. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

![Figure 3](image3.png)

**Figure 3** Effects of different concentration of chelidone on normal peripheral blood mononuclear cells incubated for 48 h tested by MTT assay. Data were expressed as mean±standard error of mean. Values were measured in three independent experiments done in triplicate. *P<0.05, **P<0.01, vs control group. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Apoptotic percentage</th>
<th>Necrotic percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>20.27±0.003</td>
<td>3.307±0.003</td>
</tr>
<tr>
<td>D₁ (22.5 μg/mL)</td>
<td>3</td>
<td>40.43±0.024*</td>
<td>4.46±0.005**</td>
</tr>
<tr>
<td>D₂ (30.0 μg/mL)</td>
<td>3</td>
<td>44.03±0.009**</td>
<td>6.45±0.007**</td>
</tr>
<tr>
<td>D₃ (37.5 μg/mL)</td>
<td>3</td>
<td>45.20±0.023**</td>
<td>10.33±0.004**</td>
</tr>
</tbody>
</table>

* **P<0.01, vs control group. LDH: lactate dehydrogenase.

2.3 Morphological changes The morphological changes of HeLa cells treated with D₁, D₂, and D₃ were also observed with respect to the positive control. Results revealed that the morphological changes (cell shrinkage and blebbing) were typical of apoptosis. Exposure to D₁, D₂ and D₃ for 48 h caused the majority of HeLa cells to shrink, round up and detach from the culture dish (Figure 4).
2.4 Fluorescence microscopic analysis

2.4.1 DAPI staining The control cells did not take positive staining with DAPI and showed no cells with visible chromatin condensation. However, with different drug treatments, cells with chromatin condensation appeared to increase in number. The D3-treated cells showed the maximum chromatin condensation (Figure 5).

2.4.2 AO-EB staining The late apoptosis was evaluated by two different methods, one of which was performed by dual staining with AO-EB and the second method was by flow cytometry-annexin V test based on phosphatidylserine determination. The cells were stained with AO-EB. The untreated culture showed intact chromatin without fragmentation and the fragmentation appeared to increase in number along with different drug treatments (Figure 6).

2.5 Result of the comet assay In the comet assay, the nuclei were intact and round, without any fragmented DNA in the control. In the treated cultures, there was an increase in comet lengths with different drug treatments, showing signs of more DNA damage with extended comet tails (Figure 7).

2.6 DNA fragmentation assay The DNA fragmentation was tested by agarose gel electrophoresis which indicated a significant increase in inter-nucleosomal DNA fragmentation of HeLa cells. When the DNA isolated from the drug-treated cells was subjected to agarose gel electrophoresis, a DNA ladder characteristic of apoptotic DNA was observed in the drug-treated cells with respect to the control (Figure 8).
2.7 CD spectral analysis  CD spectrum of CT DNA showed a positive band at 271 nm due to base stacking and a negative CD band at 248 nm due to change in helicity, which are characteristics of a typical B form DNA. Chelidonine did not have a CD spectrum of them when they were free in solution. In case of chelidonine added to CT DNA, a positive CD band appeared at 269 nm and a negative CD band at 236 nm, which showed an increase in CD molar ellipticity. This observation is an indicator of a strong binding of drug chelidonine to the CT DNA and bringing conformational change in CT DNA (Figure 9).

![Figure 9](image)

2.8 Induction of apoptosis  To confirm whether the cause of HeLa cell death induced by the drugs was apoptosis, flow cytometric analysis by annexin V-FITC/PI staining method was additionally performed. The details of the result have been summarized in Figure 10. The percentage of apoptosis was increased in the drug-treated cells gradually as compared with the control.

2.9 Effect of drugs on cell cycle arrest in HeLa cells  The drugs induced cell cycle arrest in the HeLa cells. There was an increase in cell populations in the sub-G1 and G2/M stages of the cell cycle after treatment along with the drug dose and an decrease in the cell population at G2/M stage (Figure 11).

![Figure 10](image)

![Figure 11](image)
2.10 Generation of ROS The potentials of D1, D2 and D3 to generate oxidative stress in terms of intracellular hydrogen peroxide (H$_2$O$_2$) generation were measured using the fluorescent indicator H$_2$DCF-DA. A boost in superoxide production was observed in the D1-, D2- and D3-treated cells, compared to the control cells under a fluorescence microscope and based on the FACS data (Figures 12A and 12B).

2.11 MMP The potential of D1, D2, and D3 to generate stress and collapse of MMP before cell killing and release of cytochrome c from mitochondria to the cytosol were measured using the fluorescent indicator rhodamine 123. The change in MMP was observed in the D1-, D2- and D3-treated cells compared to the control cells. From FACS data in drug-treated cells we found that there was a significant increase in fluorescence intensity as compared to the control cells (Figure 13).

2.12 Western blot analysis Denitometric data of the bands revealed that, the expression levels of p38, p53 and caspase 3 were up-regulated and PI3K, E6 and E7 levels were down-regulated upon treatment of the drugs on HeLa cells with respect to the control (Figure 14).

2.13 Measurement of protein expressions involved in apoptotic signalling pathway by RIISA Along with the doses of D1, D2 and D3, the expression levels of p38, p53, Bax, BAD, cytochrome c, caspase 9, caspase 3 and APAF-1 in the total cell lysate were up-regulated, while the expressions of PI3K, AKT, JAK3, STAT3, Bcl-2 and Bcl-xL were down-regulated with respect to the control (Figure 15).

2.14 RT-PCR analysis In semiquantitative RT-PCR analysis, the total mRNA was isolated from the control and drug-treated cells, and then they were amplified partially using the different sets of forward and reverse primers of respective genes after production of cDNA. The densitometry of the band intensity showed that, the expressions of p55, Bax, caspase 3 and PARP-1 were up-regulated and the expressions of PI3K, AKT, Bcl-2 and ICAD were down-regulated (Figure 16) after the introduction of drug with respect to control. GAPDH was taken as the housekeeping gene.

![Figure 12](image1.png) **Figure 12** ROS generation in HeLa cells observed by fluorescence microscopic observation and measured by FACS analysis
A: Fluorescence microscopic observation of ROS generation in HeLa cells (×200); B: FACS analysis of ROS generation in the D1-, D2- and D3-treated cells with respect to the control cells. Control and treated cells were incubated with H$_2$DCF-DA fluorescent probe and analyzed in a single labelling FACS system at 530 nm band pass filter using histogram plot.
D1: 22.5 µg/mL; D2: 30.0 µg/mL; D3: 37.5 µg/mL; ROS: reactive oxygen species; FACS: fluorescence activated cell sorter; PI: propidium iodide; DCFH-DA: 2',7'-dichlorodihydrofluorescein diacetate.

![Figure 13](image2.png) **Figure 13** FACS analysis of changes in MMP in HeLa cells
Control and treated cells were incubated with Rhodamine 123 fluorescent probe and analyzed in a single labelling FACS system at 530 nm band pass filter using histogram plot. D1: 22.5 µg/mL; D2: 30.0 µg/mL; D3: 37.5 µg/mL. FACS: fluorescence activated cell sorter; MMP: mitochondrial membrane potential.
Figure 14 Western blot analysis of p38, p53, caspase 3, PI3K, E6, E7 and GAPDH
The expressions of p38, p53 and caspase3 were up-regulated in the drug-treated cells with respect to the control cells whereas PI3K expression was down-regulated. GAPDH acted as a housekeeping gene. Ln1: Control; Ln2: 22.5 μg/mL; Ln3: 30.0 μg/mL; Ln4: 37.5 μg/mL. PI3K: phosphatidylinositol 3-kinases; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure 15 Up-regulated proteins and down-regulated proteins measured by ELISA
A: Up-regulated protein expression. B: Down-regulated protein expression. Data were expressed as mean ± standard error of mean. Values were measured in three independent experiments done in triplicate. * P<0.05, ** P<0.01, vs control group. D1: 22.5 μg/mL; D2: 30.0 μg/mL; D3: 37.5 μg/mL. ELISA: enzyme-linked immunosorbent assay.

Figure 16 RT-PCR analysis of p53, Bax, caspase 3, PARP-1, PI3K, AKT, Bcl-2, ICAD and GAPDH
The expressions of p53, Bax, caspase 3 and PARP-1 were up-regulated in drug-treated cells with respect to the positive control while the expressions of PI3K, AKT, Bcl-2 and ICAD were down-regulated. GAPDH acted as a housekeeping gene. RT-PCR: reverse transcription-polymerase chain reaction; PARP-1: poly adenosine diphosphate ribose polymerase-1; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase B; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; ICAD: inhibitor of caspase-activated DNase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

3 Discussion
The induction of apoptosis is a physiological process that is facilitated by the induction of chromatin condensation and fragmentation, formation of cytosolic blebbing and breakage of structural proteins\(^{[25]}\). In the present study, the drugs have been shown to induce the cellular blebbing (morphological changes study) and the increases in LDH activity, chromatin condensation (DAPI staining) and fragmentation of the DNA (DNA fragmentation assay and comet assay). The induction of late stage apoptosis was also confirmed by the AO-EB fluorescence staining. The quantitative study of apoptosis was further analyzed by flow-cytometry (annexin V-FITC conjugate staining). The collective results of these studies unequivocally pointed to the fact that chelidonine induced apoptosis in HeLa cells beyond any doubt.
In comparison with the normal cells, cancer cells generally possess high ROS activity and can tolerate higher level of ROS\cite{21}. It has been suggested that further increase in ROS exposure will push cancer cells over the tolerated ROS threshold and would drive them into apoptosis\cite{28}. ROS-induced apoptosis is highly reliant on persistent activation of pro-apoptotic MAPK pathways like p38\cite{29}. In our set of experiments, we found that the level of MAPK enzyme p38 was up-regulated with the induction of the drug and the increased ROS generation was also verified by the fluorescence microscopy (DCFH-DA staining) and FACS data. ROS is a decision-making step for the initiation of cell cycle arrest and induction of pro-apoptotic pathway by making the mitochondrial outer membrane permeable.

In our experiment, we observed that, the growth rate of the cells was reduced upon the treatment of the drug. The reduced proliferation of the cells is confirmed by the cell cycle analysis through FACS. The cell cycle analysis revealed the inside story of the growth. The increase in phases of sub-G\(_1\) and G\(_1\)/G\(_0\) cell populations after treatment adds support to the role of chelidonine in arresting the cell division, demonstrating its inhibitory effect on cancer cells.

The drug-DNA interaction study can elucidate the action potential of the drug at the molecular level. The drugs can directly bind to the CT DNA and can change the conformation of DNA. Our next goal was to identify the probable molecular mechanism of apoptosis. Members of the Bcl-2 family of proteins are known to be critical regulators of the apoptotic pathway. They act as an upstream effector molecule to suppress apoptosis\cite{30}. Bcl-2 has been shown to form a heterodimer with the pro-apoptotic Bax and might thereby neutralize its pro-apoptotic effect. Therefore, alteration in the levels of Bax and Bcl-2, namely, the ratio of Bcl-2/Bax, is the decisive factor that plays an important role in determining whether cells will undergo apoptosis leading to cell death\cite{31,32}, or be directed towards the survival pathway\cite{33}. In our study, the decrease in Bcl-2 level and increase in Bax expression at both RNA and protein levels in HeLa cells were observed. Hence, the ratio of Bcl-2/Bax was altered in favour of apoptosis. Therefore, our results suggest that up-regulation of Bax and down-regulation of Bcl-2 may be indicative of the actual molecular mechanism through which chelidonine induces apoptosis.

Mitochondria contain several apoptogenic proteins like Bcl-2 family proteins and cytochrome c. The change in membrane potential helps in releasing these proteins. In our studies the change in MMP relates to the release of proteins like cytochrome c and Bcl-2 family proteins. Mitochondria are known to contain and release protein such as cytochrome c\cite{34} that is involved in the apoptotic cascade by activating caspases, resulting in the appearance of apoptosis, and our study confirms the induction of this mechanism by the drug.

It is widely considered that, the release of cytochrome c and activation of Apaf-1 induce the cascade of caspases, which are involved in apoptosis\cite{35}. In our present study, we observed up-regulation of caspase 9 and caspase 3, which in turn can induce apoptosis. In the downstream, caspase 3 activates the chromatin-cleaving elements like poly PARP\cite{36} and deactivates ICAD\cite{37}. In our present study, the PARP-1 level increased and the ICAD level decreased, which would confirm the reason for chromatin fragmentation in the chelidonine-treated HeLa cells, which portray the conclusion of the process of apoptosis.

The dysregulation of the PI3K/AKT signalling pathway can lead to an alteration of all the aspects of cell physiology that comprise the hallmark of cancer. Cell survival is influenced by AKT through a variety of effector proteins including inhibition of the pro-apoptotic BAD in the Bcl-2 family\cite{38}. The JAK3-STAT3 pathway up-regulates another Bcl-2 family protein, Bcl-XL that in turn leads to an anti-apoptotic stage in the formation of tumour cells\cite{39}. On the other hand, p53 regulates the cell apoptosis by balancing the Bcl-2/Bax ratio. The activated p53 also induces apoptosis by up-regulating the level of Bax\cite{40}.

The PI3K/AKT pathway, JAK3-STAT3 pathway and p53 pathway are activated or down-regulated in the downstream of various extracellular signals like p38 and activation of this signalling pathway impacts a number of cellular processes including cell growth, proliferation, survival\cite{41} and apoptosis\cite{42}. We found that, treatment of HeLa cells with D1, D2 and D3 resulted in decreases of the expressions of AKT, PI3K, JAK3, and STAT3 and increases in the expressions of p38 and p53.

E6 and E7 are the HPV proteins associated with cervical cancer. E6 binds to and inactivates the tumor suppressor p53, while by associating with pRb, E7 prevents its binding with E2F transcription factors, thereby promoting cell cycle progression. Therefore, increased expressions of E6 and E7 lead to the malignant transformation of the host cells, deregulation of the cell cycle and tumor formation\cite{43}. To assess the effect of chelidonine on HeLa cells, Western blot analysis was used to investigate the expression levels of E6 and E7 oncoproteins. Results of our present study reveal that with increasing concentrations of chelidonine, both E6 and E7 expression levels were significantly down-regulated. Thus, our findings supported the above contention where it was evident that, chelidonine down-regulates E6 and E7 expressions, up-regulates p53 expression and arrests cell cycle at sub-G\(_1\) and G\(_{0}\)/G\(_{1}\) stages in HeLa cells.

We observed an elevated level of p38 expression triggered by the drug. We also observed up-regulation
of the downstream protein p53 and down-regulation of AKT and STAT3, which could be a part of the cascade of gene actions triggered by the p38, which can only be confirmed after further stepwise inhibitor studies. However, over-expression of p38 is already known to block the cell cycle\cite{36} and to enhance the activity of p53 transcription factor\cite{37}. Over expression of p38 is also reported to inhibit the expression of AKT\cite{45} and STAT3\cite{46}. The down regulation of AKT, which we also observed in this study, has been reported to inhibit the proliferation of cells. Thus, we suspect that the drug triggered the up-regulation of p38, and as a consequence of that, there was an up-regulation of p53 that triggered over expression of the pro-apoptotic genes at one hand, and down-regulation of AKT and STAT3, resulting in inhibition of cell proliferation on the other.

4 Conclusion

In conclusion, we can suggest that chelidonine had great anticancer potentials in an in vitro system. The mechanism through which it achieved its goal was more by inducing apoptosis in the HeLa cells through activation of p38-p53-dependent pathway on one hand, and also through its inhibitory influence on the proliferating ability of the cells by down-regulating the JAK-STAT and AKT pathway on the other.

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6 Competing interests

The authors declare that they have no competing interests.

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从白屈菜乙醇提取物中分离出的白屈菜碱通过p38-p53和PI3K/AKT信号转导通路促进HeLa细胞凋亡

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目的：研究从白屈菜乙醇提取物中分离出的白屈菜碱在诱导HeLa细胞凋亡中的作用及参与其作用的主要信号转导通路。

方法：细胞先以不同浓度白屈菜碱处理48 h，用噻唑蓝法分析确定半数致死量（median lethal dose, LD₅₀）。用4’,6-二脒基-2-苯基吲哚染色，追踪分析核染色以及DNA损伤和碎片的形态学变化，并用流式细胞术分析检测活性氧（reactive oxygen species, ROS）的产生以及细胞周期阻滞和线粒体膜电位的变化。用双色光谱分析寻找白屈菜碱和小牛胸腺DNA可能的相互作用。用逆转录聚合酶链反应和蛋白免疫印迹法测定p38、p53、蛋白激酶B（protein kinase B, AKT）、磷脂酰肌醇3-激酶（phosphatidylinositol 3-kinases, PI3K）、Janus激酶3（Janus kinase 3, JAK3）、信号转导及转录激活因子3（signal transducer and activator of transcription 3, STAT3）等的mRNA和蛋白质表达，以及E6、E7癌基因和促凋亡基因、抗凋亡基因的mRNA和蛋白质表达。

结果：根据白屈菜碱的LD₅₀（30 µg/mL），选定3种实验剂量，即22.5, 30和37.5 µg/mL。结果显示，白屈菜碱抑制了HeLa细胞增殖，诱发其细胞凋亡，表现为ROS的产生，细胞亚G₁和G₀/G₁周期阻滞，线粒体膜电位变化和DNA碎片产生。双色光谱分析结果显示白屈菜碱和小牛胸腺DNA间存在有效的相互作用。信号转导的研究显示白屈菜碱通过上调p38、p53和其他促凋亡基因的表达，以及下调AKT、PI3K、JAK3、STAT3、E6和E7及其他抗凋亡基因的表达，有效诱发细胞凋亡。

结论：从白屈菜中分离出的白屈菜碱能通过改变p38-p53及AKT/PI3激酶信号转导通路有效地诱发HeLa细胞凋亡。

关键词：白屈菜碱；白屈菜；抗肿瘤药；植物；信号转导；细胞凋亡；HeLa细胞