Anticancer potential of *Conium maculatum* extract against cancer cells *in vitro*: Drug-DNA interaction and its ability to induce apoptosis through ROS generation

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ABSTRACT

Objective: *Conium maculatum* extract is used as a traditional medicine for cervix carcinoma including homeopathy. However, no systematic work has so far been carried out to test its anti-cancer potential against cervix cancer cells *in vitro*. Thus, in this study, we investigated whether ethanolic extract of conium is capable of inducing cytotoxicity in different normal and cancer cell lines including an elaborate study in HeLa cells. **Materials and Methods:** Conium's effects on cell cycle, reactive oxygen species (ROS) accumulation, mitochondrial membrane potential (MMP) and apoptosis, if any, were analyzed through flow cytometry. Whether Conium could damage DNA and induce morphological changes were also determined microscopically. Expression of different proteins related to cell death and survival was critically studied by western blotting and ELISA methods. If Conium could interact directly with DNA was also determined by circular dichroism (CD) spectroscopy. **Results:** Conium treatment reduced cell viability and colony formation at 48 h and inhibited cell proliferation, arresting cell cycle at sub-G stage. Conium treatment lead to increased generation of reactive oxygen species (ROS) at 24 h, increase in MMP depolarization, morphological changes and DNA damage in HeLa cells along with externalization of phosphatidyl serine at 48 hours. While cytochrome c release and caspase-3 activation led HeLa cells toward apoptosis, down-regulation of Akt and NFkB inhibited cellular proliferation, indicating the signaling pathway to be mediated via the mitochondria-mediated caspase-3-dependent pathway. CD-spectroscopy revealed that Conium interacted with DNA molecule. **Conclusion:** Overall results validate anti-cancer potential of Conium and provide support for its use in traditional systems of medicine.

Key words: Apoptosis, *Conium maculatum*, drug-DNA interaction, proliferation, reactive oxygen species

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INTRODUCTION

Conium maculatum is an extremely poisonous flowering weed, known as Hemlock and belongs to the family Apiaceae. Conium contains several pyridine alkaloids like coniine, *N*-methylconiine, conhydrine, pseudoconhydrine and gamma-coniceine, precursors of some other hemlock alkaloids.[1] The structures of these alkaloids are shown in Figure 1a. Among these, the most notable one is coniine, the properties of which are similar to nicotine. It disrupts the functions of the central nervous system by binding with nicotinic acetylcholine receptors.[2,3] Though this plant is highly toxic in nature, its extract had been used as a traditional remedy for different diseases since a long time.[4] As for example, Conium is the main remedy for prostate gland and swelling of the testis. In homeopathy, it is used as a remedy for breast cancer and cancer of cervix uteri,[4] but its action has not yet been scientifically validated except for the report that it can inflict DNA damage by generating reactive oxygen species (ROS).[5] In this study, we contemplate to elucidate the probable mechanism of action of the drug in inducing apoptosis in the cervix cancer cell line HeLa.

In recent years, DNA-targeted therapy has gained much importance and a drug's capacity to interact with DNA has been implicated to its capacity to hinder the process of cellular replication and protein synthesis, resulting eventually in cell growth arrest and apoptosis.[6] In this

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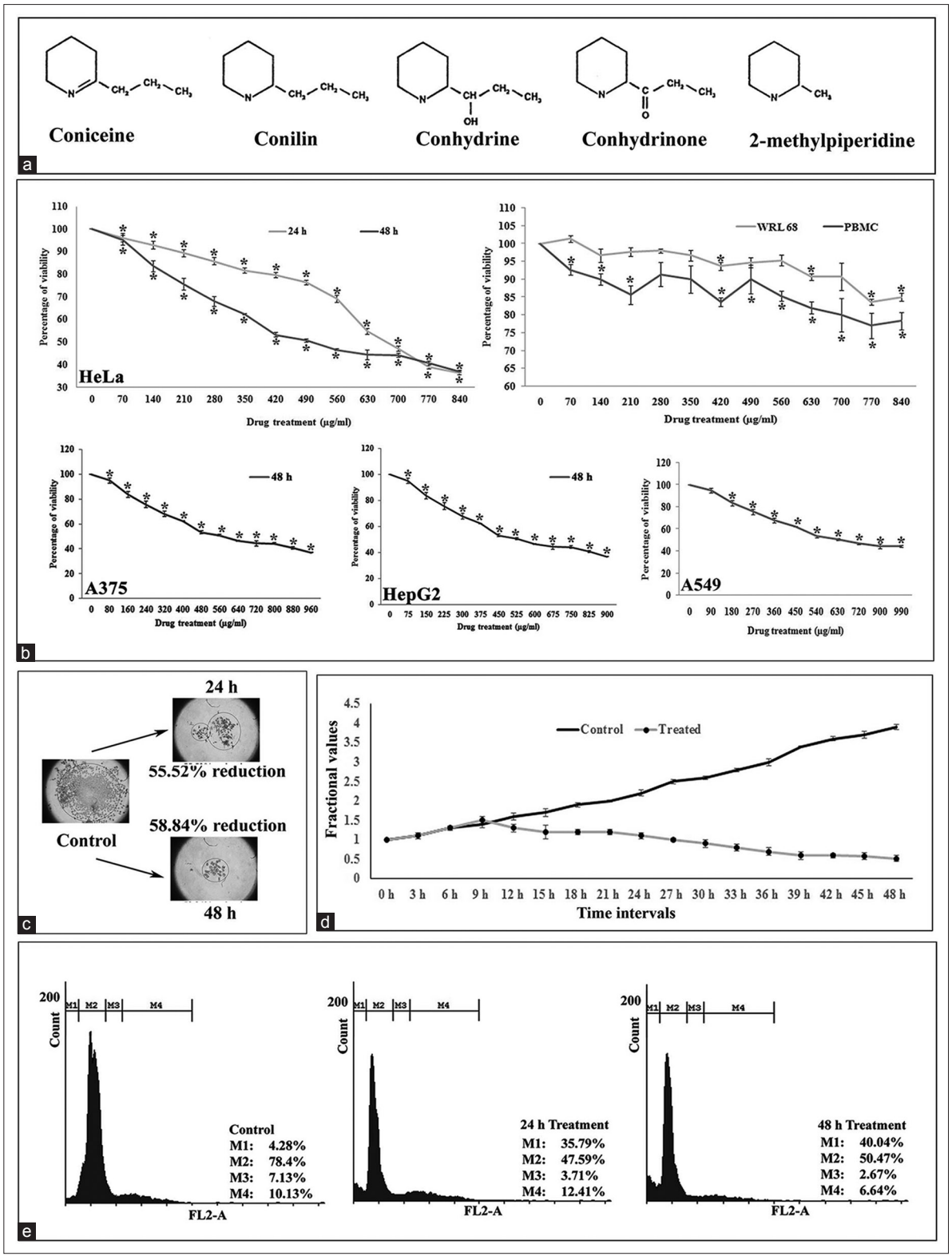

Figure 1: (a) Chemical structures of major alkaloids present in Conium. (b) Percentage of cell viability: 70-840 µg/ml of drug was supplemented to A375, A549, HepG2, WRL-68 and PBMCs, and incubated for 48 h. Further 70-840 µg/ml of drugs were supplemented in the culture of HeLa for 24 and 48 hours. MTT assay was performed for all the cases to assess the percentage of cell viability [Significance **P*<0.05 vs. control group]. (c) Clonogenic assay: Treatment with Conium reduces colony formation ability of HeLa cells. (d) Proliferation assay: This indicated the reduction in proliferative property of HeLa cells upon treatment with Conium of dose 450 µg/ml then the control plates at different time intervals. Lowest proliferation achieved at 48 h of treatment. (e) Cell cycle analysis: In the treated sets (450 µg/ml Conium), there was a sharp increase in cell population at M1=sub-G1 state of cell cycle with respect to control set, indicating the induction of DNA fragmentation after 24 and 48 hours

study, we tried to evaluate whether Conium has the capacity to interact with either naked calf thymus DNA (ct-DNA) or cellular DNA of HeLa cells, which had not been studied earlier.

Herbal extracts are considered a rich source of alkaloids and flavonoids.[7] They have the capability to generate ROS. An excessive accumulation of ROS beyond toleration limit generally pushes the cancer cells toward apoptotic cascade.[8] ROS also depolarizes mitochondrial membrane potential (MMP) and induces apoptosis.[9] Since Conium is a rich source of alkaloids[10,11] which have been reported to have anti-cancer properties, we became interested to examine if it has any such anti-cancer effects against HeLa cells and can produce ROS and cause mitochondrial membrane de-polarization.

The events of cell death and cell growth are controlled by certain signaling proteins.[12] Therefore, it is expected that any anti-proliferative drug should have the nature to down-regulate the activities of these signal molecules to hinder the process of cancer cell growth.[13] Alternatively, apoptosis is closely mediated by different anti-apoptotic and apoptogenic proteins. Caspase cleavage generally initiates apoptosis followed by induction of DNA fragmentation.[14] In this study, we tried to evaluate the modulating capability of Conium on certain signal proteins related to apoptosis and proliferation; to our knowledge, this approach had not been made in any earlier study.

Thus, in the present study the hypotheses to be tested were whether: (i) Conium has capability to induce cytotoxicity in HeLa cells and also has a hindering effect on proliferation; (ii) Conium acts as a DNA-binding agent, producing conformational changes in ctDNA and cellular DNA of drug-treated HeLa cells; and (iii) Conium can accumulate ROS and depolarize MMP by modulating certain cell signaling proteins related to apoptosis and proliferation.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), RPMI-1640 and penicillin, streptomycin, neomycin (PNS) antibiotic were purchased from HiMedia (India). Fetal bovine serum (FBS), trypsin and ethylene di-amine tetra-acetic acid (EDTA) were obtained from Gibco BRL (USA). Tissue culture plastic wares were purchased from Tarson (India). Acridine orange (AO) and ethidium bromide (EB) were purchased from SRL (India). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dichloro-dihydrofluoresceindiacetate (H_2DCFDA), 4×,6-diamidino-2-phenylindole dihydrochloride (DAPI) and rhodamine 123 and secondary antibodies were obtained from Sigma (USA). Annexin V-fluorescein isothiocyanate (FITC) and primary antibodies were obtained from Santacruz Biotechnology Inc. (USA).

Conium extract

Ethanolic extract of *C. maculatum* was procured as homeopathic mother tincture from Boiron Laboratory®, Lyon, France, which was prepared by using 65% ethanol as solvent, adopting the guidelines of European Pharmacopeia 7thedition (http://www.pharmabooks.com.br/livros/images/livros/Index_7th_Edition_70.pdf).

Cell culture

HeLa, A375, HepG2, A549 (all of cancer cell lines) and WRL-68 (of normal liver) cells were collected from National Centre for Cell Science, India. The cells were maintained in the humidified incubator (ESCO, Singapore) with ambient oxygen and 5% carbon dioxide level at 37°C. Cells were cultured in DMEM with 10% heat-inactivated FBS and 1% PSN. Cells were harvested with 0.025% Trypsin-EDTA in phosphate buffer saline (PBS), were plated at required cell numbers and allowed to adhere for required time before treatment.

Peripheral blood mononuclear cells (PBMC; normal blood cells from healthy mice) were immediately isolated by gradient centrifugation in Ficoll-Hypaque by a standard method and washed twice with phosphate buffered saline (PBS) and once more with RPMI-1640.

Cell viability assay

HeLa, A375, HepG2, A549, WRL-68 and PBMCs were dispensed into 96-well flat bottom micro-titter plates (Tarson, India) at a density of 1 × 10^3 cells per well. Cell were treated with various concentrations of Conium (70 to 800 μg/ml) and incubated for 48 hours. MTT solution (10 μM) was then added to each well and incubated for 3 h at 37°C. Insoluble formazan crystals formed were dissolved in 100μl acidic isopropanol and optical density was measured at 595 nm in an ELISA reader (Thermo scientific, USA).[15] Of all the cancer cell lines, cytotoxicity of Conium was found to be most conspicuous and overwhelmingly greater in HeLa than in the other cancer cell lines. Further, it also did not show profound cytotoxicity on normal cells like WRL-68 and PBMCs. Therefore, HeLa cells were preferred over the other cancer cells as most suitable materials for conducting all other experiments related to its probable mechanism of action and the signaling pathway involved in inducing apoptosis.

Selection of doses

Three different doses were selected depending on MTT assay result namely, D1 = 150 μg/ml, D2 = 300 μg/ml

and D3 = 450 μg/ml. Before the experimental treatment the drug was diluted in the DMEM media. The positive control (vehicle of drug) set received only diluted ethanol (0.48% after dilution), while the negative control received neither drug nor ethanol. As the positive control did not show any palpable difference in results as compared to the negative control, we abandoned the negative control and maintained the positive control only for comparison of results. Incubation time of drug treatment was taken depending upon the requirement of the specific experiment.

Colony formation assay

HeLa cells were assayed for the cytotoxic effects of Conium after cell survival, according to the established methods of performing the clonogenic assay. Sub-confluent cultures were exposed to drugs for 6 hours. Then the cells were washed with PBS (phosphate buffered saline) preheated to 37°C, trypsinized and plated in 6-well plates (100 cells/well). After 12 days of incubation in complete culture medium, the colonies were stained with Giemsa's stain after fixation with 2% para-formaldehyde.[16]

Proliferation assay

To perform the cell proliferation assay, treated cells were harvested after different intervals between 0 to 48 h, washed twice with PBS and trypsinized. The cell suspension was then transferred to a hemocytometer for cell counting. This procedure was repeated for all samples at each time point, and the experiments were repeated three times. After analysis of data a cell proliferation histogram was obtained.

Cell cycle analysis

Propidium iodide (PI) was used for cell cycle analysis. Cells were treated with 450 μg/ml of Conium for 24 and 48 hours. Cells were then fixed with 70% ethanol and made RNA free. 5 μM of PI were added to them and incubated for 20 min in dark. Fluorescence intensity was measured using FL-2H for PI.

Detection of reactive oxygen species accumulation

ROS accumulation was assayed quantitatively after incubation of 0, 6, 12, 18, 24, 30, 36, 42 and 48 h, respectively, with 450 μg/ml Conium; the cells were fixed with 70% chilled ethanol and then incubated with 10 μM DCHFDA for 30 min in cold and dark. Fluorescence intensity was measured by flow cytometry using FL-1H filter and data were analyzed by using Cyflogic software.

Analysis of changes in mitochondrial membrane potentials

MMP was measured both qualitatively and quantitatively as described by Bishayee *et al.*[6] After 48 h incubation, control and treated (150, 300, and 450 μg/ml of Conium) cells were fixed with 2% paraformaldehyde and then incubated with 10 μM rhodamine 123 for 30 min at 37°C in dark. Cells were immediately analyzed using florescence microscopy. Secondly, cells were fixed in 70% ethanol and incubated in 10 μM rhodamine 123 for 30 min at 37°C in dark. To determine the MMP the fluorescence intensity of rhodamine123 was assessed by flow cytometry using FL-1H filter (BD FACS Calibur, USA) and data were analyzed using Cyflogic software.

Cell morphological analysis

For this study, HeLa cells were treated with 150, 300 and 450 μg/ml of Conium, respectively. Control set did not receive any drug. After 48 h incubation, HeLa cells were observed under an inverted phase contrast microscope (Leica, Germany), equipped with a digital camera and photographs were taken.

Nuclear morphology analysis

DAPI and AO/EB Staining: One control and three drug doses (150, 300, and 450 μg/ml of Conium) were selected for this experiment. After 48 h treatment, cells were fixed with 2% paraformaldehyde. Then cells were stained with DAPI and AO/EB at 10 μM concentration each and observed under fluorescence microscope (Leica, Germany).

Annexin V/PI assay

Cells were treated with 450 μg/ml of Conium for 24 and 48 hours. Cells were then fixed with 70% ethanol and made RNA free. 10 μM of Annexin V and 5 μM of PI were added to them and incubated for 20 min in dark. Fluorescence intensity was measured using FL-1H and FL-2H for annexin V and PI, respectively.

DNA fragmentation assay

Control and treated (150, 300, and 450 μg/ml of Conium, respectively) HeLa cells were washed in PBS and incubated with DNA Lysis buffer (10 mM Tris, 400 mM NaCl, 1 mM EDTA, 1%Triton X-100, RNase (0.2 mg/ml) and proteinase K (0.1 mg ml)) overnight, then centrifuged at 1200 g at 4°C. Supernatants were then mixed with phenol-chloroform-isoamyle mixture (25:24:1) and bi-layered mixture was centrifuged at 1500 g, 4°C for 15 minutes. DNA was then precipitated using 100% ethanol, from the aqueous layer and dissolved in 20 μl of Tris–EDTA buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA). Extracted DNA was further purified using proteinase K and RNase A to subtract excess proteins and RNAs, respectively. Purified DNA was separated using 1.5% agarose gel electrophoresis and bands were visualized under a UV trans-illuminator, followed by digital photography.

Western blot analysis

HeLa cells were seeded into 75-mm plates (Tarson, India) at a density of 1×10^5 cells per well. Cell were treated with various concentrations of Conium (150, 300, and 450 μg/ml, respectively) and incubated for 48 hours. Equal amounts of protein (50 μg) were run on 12.5% SDS–PAGE and electrophoretically transferred to PVDF membrane. After blocking with 3% BSA, the membranes were separately incubated with specific primary antibodies (p53, Bcl-2, Bax, TNF-α, PARP, Caspase3, Akt, pAkt, NFKβ and GAPDH) overnight at 4°C. The membrane was again incubated for 2 h with the ALKP conjugated secondary antibody. BCIP-NBT was used as developer and quantification of proteins was done by densitometry using image J software.[17]

Assessment of cytochrome c activity by indirect ELISA

For this experiment, HeLa cells were treated with different doses of Conium (150, 300 and 450 μg/ml) and incubated for 48 hours. The assay was done according to the manufacturer's protocol (Santa Cruz Biotechnology Inc, USA). Protein activity level of Cytochrome c was measured by using ELISA reader. PNPP (p-nitrophenyl phosphate) was used as a color developer and color intensity was measured at 405 nm against blank. G3PDH served as the housekeeping gene in this assay.[18]

Drug–DNA interaction

For assessment of conium and nuclear DNA interaction, two modes of study were performed; first interaction was checked on naked calf thymus cell DNA (ctDNA) with drug concentration of 450 μl/ml using untreated ctDNA as control; secondly, to measure interaction of drug with DNA within cells, incubation was performed with conium at concentrations of 450 μl/ml for 3 hours. After 3 h incubation, cells were collected and their DNA was extracted and purified using GeneiPure Mammalian Genomic DNA Purification Kit, India. Collected DNA was used to analyze CD spectra for determination (JASCO J720, Japan) of drug-DNA interaction, if any, using Origin 8 Pro software.[19]

Statistical analysis

Statistical analysis was performed by one-way ANOVA with LSD *post hoc* tests, using SPSS.14 software to identify if the differences were significant among the mean-values of different groups. Results were expressed as Mean ± SE (Standard Error). $*P < 0.05$ was considered as significant.

RESULTS

Conium treatment reduced cell viability and colony formation ability of HeLa cells

In our initial trial with different cancer cell lines, Conium was found to reduce viability of A375, HepG2 and A549. 50% cell death occurred at the dose of 657.7 ± 6.8, 616.6 ± 6.3 and 730.1 ± 6.9 μg/ml on A375, HepG2 and A549, respectively, for 48 h treatment [Figure 1b].

However, the cytotoxicity was found to be strikingly greater against HeLa cells. The MTT result revealed that the cytotoxicity of Conium palpably increased at 48 h, greater than that observed at 24 h of treatment. The reduction in viability of HeLa cells was dose-dependent like that of other cell lines we used. For 24 h drug incubation, the percentage of viability was reduced to 36.48 ± 1 for drug dose of 840 μg/ml and 50% reduction in cell viability occurred at 722.6 ± 8.0 μg/ml. For 48 h drug incubation, the percentage of cell viability was reduced to 36.93 ± 0.2 for drug dose 840 μg/ml and 50% reduction in cell viability occurred at 575.5 ± 5.0 μg/ml [Figure 1b].

MTT results for the normal cell lines (WRL-68 and PBMCs) revealed that there was a minimal cytotoxicity produced by Conium at 48 h of treatment [Figure 1b]. In case of WRL-68, the viability percentage was reduced to 85 ± 1.1 by 840 μg/ml Conium treatment for 48 h and for PBMCs the viability percentage got declined at higher doses and was 78.3 ± 2.4% at 840 μg/ml Conium treatment for 48 hours.

The clonogenic assay's result pointed out that Conium could reduce the colony forming ability of HeLa cells. After 24 and 48 h conium treatment (450 μg/ml) colony forming ability was reduced to 55.5% and 58.8%, respectively, as compared to control (100%). These findings would suggest that Conium had ability to prevent colony forming of HeLa cells [Figure 1c].

Conium treatment reduced cell proliferation and caused cell cycle arrest

Conium treatment reduced the cell growth with respect to control sets. In early hours, treatment with Conium did not affect the cellular proliferation but after 9th h of treatment, cell proliferation gradually decreased and the lowest proliferation was achieved at 48 h of treatment [Figure 1d].

Indication of cell cycle arrest was found in Conium-treated cells. In the treated sets (450 μg/ml Conium), there was a sharp increase in cell population at sub-G1 state with respect to control set, indicating thereby that the induction of DNA fragmentation was set in after 24 and 48 h [Figure 1e].

Conium treatment initiated ROS accumulation in HeLa cells

DCHFDA assay result revealed that Conium treatment initiated accumulation of ROS. The sudden shift of the histogram was observed in between 18 and 24 h of the treatment [Figure 2a].

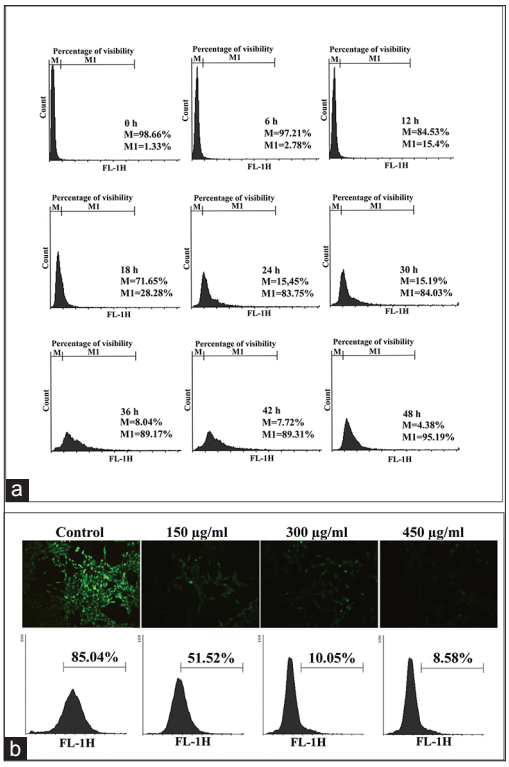

Figure 2: (a) ROS generation: This time-dependent study revealed the accumulation of ROS by the drug treatment (450 µg/ml Conium). The sudden shift of the histogram was observed in between 18 and 24 h of the treatment, indicating the accumulation of ROS. (b) Mitochondrial membrane potential: Fluorescence intensity reduced gradually with respect of Conium doses, indicating gradual depolarization of MMP. FACS studies revealed shift of picks was toward the y-axis compared to control, supporting results of microscopic study

Conium treatment depolarized mitochondrial membrane potentials

The fluorescence images revealed a decrease in mitochondrial membrane polarization. The depolarization was dose dependent, and maximum depolarization was observed with 450 µg/ml of Conium treatment [Figure 2b].

Conium treatment induced morphological changes in HeLa cells, with nucleosomal fragmentation

Morphological changes were observed in HeLa cells on treatment with Conium, with rounding off of the cytoplasmic periphery along with gradual detachment of cells from substrate. Features included cell membrane blebbing and cell shrinkage in Conium-treated cells [Figure 3a]. Fluorescence intensity was observed in AO/EB stained cells, with respect to control cells [Figure 3b]. Nuclear condensation and color change from green to reddish orange of fragmented nuclear membranes represented induction of apoptosis in treated cells as compared to control. Nucleosomal fragmentation was confirmed by DAPI staining [Figure 3c], fragmentation being of greatest magnitude with 450 µg/ml Conium dose.

Conium induced plasma-membrane externalization of phosphatidyl serine with initiation of DNA fragmentation in HeLa cells

Quantitative measurement of apoptosis was done through the estimation of externalization of phosphatidyl serine moiety. The FACS analysis with annexin V/PI was done at both 24 and 48 h of Conium treatment (450 µg/ml). Cells showed distinct positive binding with annexin V when treated with Conium-treated group, indicating externalization of phosphotidyl serine to the cell surface [Figure 3d].

The result of DNA fragmentation analysis also suggested initiation of cell DNA fragmentation in Conium-treated group [Figure 3e].

Conium treatment modulated expression of different proteins related to cell proliferation and apoptosis in HeLa cells

Treatment with Conium up-regulated the expression of certain proteins related to apoptosis. The p53 expression was up-regulated by 19%, 37% and 45% with the drug dose of 150, 300 and 450 µg/ml, respectively. Bax expression was hiked by 12%, 23% and 24% at the same drug dose and TNF-α activity also enhanced by Conium treatment by 72%, 82% and 86% with 150, 300 and 450 µg/ml dose, respectively. Conium treatment enhanced the PARP protein cleavage in a dose-dependent manner. With the PARP cleavage, caspase 3 activity was also increased by ~3 folds at the highest drug dose. The events of PARP cleavage and caspase 3 activation would indicate the induction of apoptosis in Conium-treated cells [Figure 4a].

There were some proteins, whose activity got down-regulated by conium treatment. They have the property to hinder apoptosis. Native Akt along with its activated form pAkt expression were down-regulated by conium treatment. The pAkt/Akt ratios decreased and were 1, 1.02, 0.79 and 0.76 for control, 150, 300 and 450 µg/ml of Conium treatment, respectively. With Akt, another anti-apoptotic protein, Bcl-2 expression was also down-regulated by ~23% and NFkB expression also down-regulated by ~28% with Conium treatment in HeLa cells [Figure 6]. GAPDH served as loading control [Figure 4a].

The ELISA assay on cytochrome c pointed out its increased activity in Conium-treated cells in a dose-dependent manner [Figure 4b].

Conium-induced circular dichroism spectral changes in both ct-DNA and HeLa DNA

CD spectroscopic results indicated that Conium could interact with native B-conformation of DNA of both ct-DNA [Figure 5a] and nuclear DNA [Figure 5b] of Conium-treated HeLa cells. The ct-DNA and HeLa nuclear

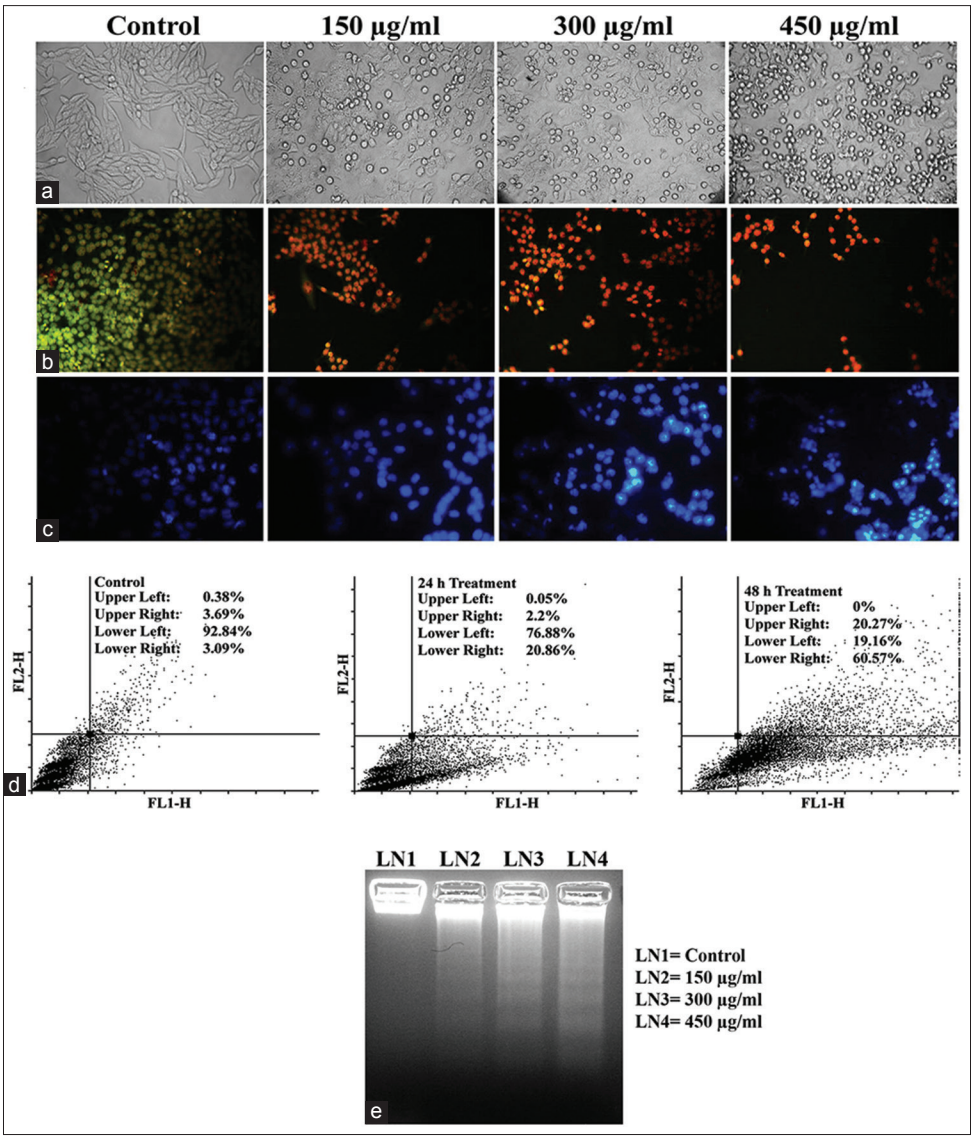

Figure 3: Apoptosis determination: The HeLa cells were treated with 150, 300, and 450 µg/ml of Conium for 48 hours. (a) Morphological analysis: result showed cell membrane blebbing and cell shrinkage in Conium-treated cells. (b) AO/EB staining: nuclear condensation and color change from green to reddish orange of fragmented nuclear membranes represented induction of apoptosis of treated cells compared to control. (c) DAPI staining: Maximum amount of nucleosomal fragmentation was observed in 450 µg/ml Conium dose, indicated by brighter fluorescence intensity. (d) Annexin V assay: Presence of apoptotic cells were observed by 48 h of treatment. Dot plot suggested the presence of early apoptotic cells by conium treatment by 24 and 48 h [Upper left= dead cells, lower left= live cells, upper right= late apoptotic cells and lower right= early apoptotic cells]. (e) DNA fragmentation assay: Presence of fragmentation was observed in Conium-treated cells by 48 h of treatment. Fragmentation was most at highest dose

DNA showed a positive band at 258 and 255 nm and a negative band at 366 and 310 nm, respectively. In Conium-treated ct-DNA and HeLa nuclear DNA, the peak shifts for positive bands were at 262 (+4) and 255 (0), respectively and for negative bands were at 375 (+9) and 303 (-7). Such changes might be due to result from structural alterations induced by Conium to DNA's double-helical structure.

DISCUSSION

Based on our initial results that showed cell viability of HeLa cells to be strikingly reduced, we chose to carry on further studies on HeLa cells for evaluation of the mechanism of action of Conium including its ability to interact with the DNA to induce apoptosis in them. Incidentally, DNA-targeted therapy has recently been proved to be an effective measure for developing anticancer drugs through its inhibiting role in cell proliferation.[20-22] CD-spectrum analysis revealed a significant alteration by Conium to DNA in a groove-binding mode. Thus, cytotoxicity could be explained by assuming that it could inhibit normal process of DNA synthesis and pushed cancer cells toward apoptosis. The present study also revealed that conium up-regulated ROS activity at early hour

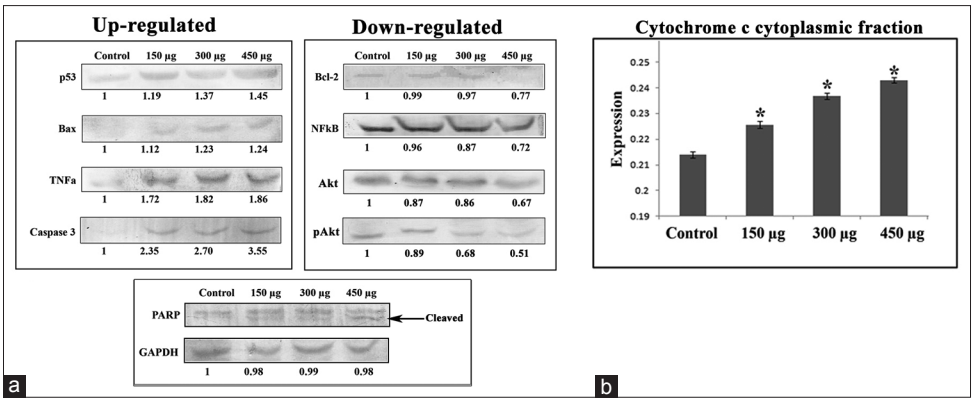

Figure 4: (a) Western blotting analysis: By Conium treatment, p53, Bax, TNF-α, and caspase 3 activities were up-regulated and Bcl-2, NFkB, Akt and pAkt expression was down-regulated. Cleaved PARP expression was increased by Conium treatment for 48 hours. GAPDH was used as loading control. (b) ELISA analysis: Cytochrome-c activity was up-regulated at cytoplasmic fraction by drug treatment for 48 hours. The x-axis represents the expressing activity of cytochrome c in terms of optical density values. Significance **P*<0.05 vs. control group

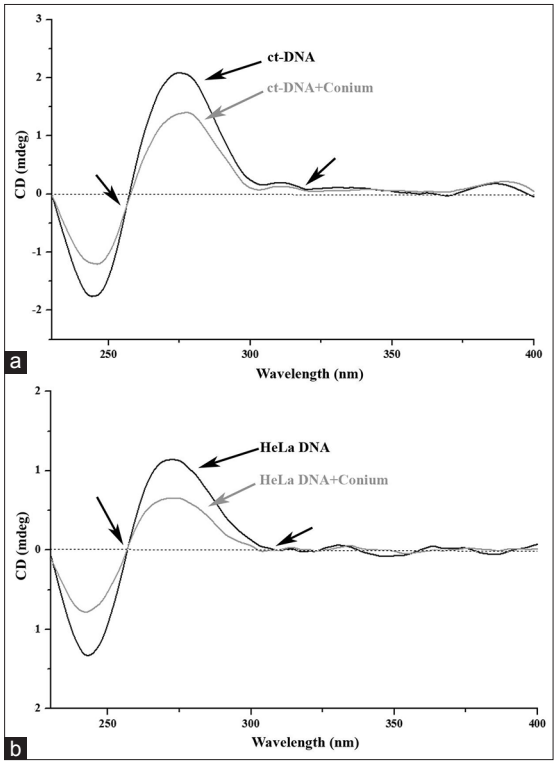

Figure 5: Drug-DNA interaction: Conium can interact with ct-DNA (a) and with cellular DNA within 3 h of drug administration

in HeLa cells. This event could manifest morphological changes and DNA fragmentation in Conium-treated cells. Conium-treatment reduced the viability and colony formation in a time/dose-dependent manner with the formation of hypo-diploid cells and increase in cell population at annexin V-positive/PI-negative quadrant, that confirmed the ability of conium to induce apoptosis of HeLa cells within 48 h of treatment; concomitantly, the event like growth rate of the cells was reduced with the increase in cell population at sub-G1 phase after conium treatment, which adds further support to the role of conium in arresting the cell division, demonstrating its growth inhibitory effect on cancer cells. Incidentally, proliferation is mainly enhanced by the protein called pAkt/Akt.[23] The administration of the drug brought down the level of pAkt/Akt and presumably produced thereby a condition that resulted in the reduction in cell growth of the cancer cells.

The accumulation of ROS in the cells could also be the cause for a reduced rate of proliferation of the cells. These events could lead to the manifestation of morphological changes of detaching cells showing condensed and fragmented chromatin. The results of MTT assay also showed that the viability of the cancer cells was gradually reduced with the increase of the drug dose but such a condition was not observed in case of WRL-68 and PBMCs. The analysis of the apoptotic phenomenon was further confirmed by the data of DAPI, AO/EB staining, DNA fragmentation and AnnexinV/PI assay. In our findings, an increase in the population of cells undergoing an early-apoptotic state (as manifested by cells with bright orange chromatin with highly condensed and fragmented cell boundaries) with fragmented cellular DNA was noted in the different drug-treated series. Thus, there was clear evidence that the drug could bring about cellular events promoting the apoptotic activity in the cells within 48 h of treatment.

Cell cycle arrest and growth inhibition may induce a greater sensitivity to the cell of having more tumor suppressor proteins like p53.[13] p53 expression was up-regulated along with the cell cycle arrest and growth inhibition. With the arrest of growth, the expression of Akt/pAkt, an important cell differentiation and proliferative molecule,[12] got down-regulated. One hypothesis to explain this event could be that a p53-Akt crosstalk took place after the drug induction.

Accumulation of ROS and depolarization of MMP are concurrent processes,[24] resulting in discharge of cytochrome c into the cytoplasm,[25] which initiates apoptosis via the intrinsic pathway.[26] In our study, the depolarization of MMP occurred with the increment of cytosolic cytochrome c upon the drug treatment. This would indicate that early elevation of ROS played a regulatory mechanism to initiate apoptosis in mitochondrial-dependent manner.

The expression of Bax is up-regulated by the tumor suppressor protein p53, and Bax has been shown to be involved in p53-mediated apoptosis. Bax was found in the cytosol, but upon initiation of apoptotic signaling, it underwent a conformational shift to become associated with mitochondrial membrane. This results in the release of cytochrome c and other pro-apoptotic factors from the mitochondria leading to activation of caspases.[26] On the other hand, Bcl-2 has been shown to form a hetero-dimer with the pro-apoptotic Bax and might thereby neutralize its pro-apoptotic effect. Therefore, alterations in the levels of Bax and Bcl-2, i.e. 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.[27] Our findings showed that the level of Bcl-2 was decreased with an increase in Bax level, indicating activation of the intrinsic pathway of apoptosis (type 1 cell death).

The ROS has the involvement of the up- and down-regulation of some pro- and anti-inflammatory proteins like NFkβ and TNF-α. The TNF-α is a factor or receptor, which in turn is activated by the down-regulation of NFkβ.[28] This activated TNF-α in turn activates caspase 3 at downstream[29] level. Our results showed the up-regulation of TNF-α after the administration of Conium and further activated caspase 3 at its downstream, which pushed HeLa cells toward apoptosis.

CONCLUSION

From results of present study, we can deduce that Conium has the potential to interact with the DNA and thus hinders in the process of cell proliferation and cell cycle; it accumulates ROS in HeLa and thus depolarizes MMP which initiates cytochrome-c discharge into the cytosol. The actual mechanism of apoptosis lies in the up- and down-regulations of some proteins. The up-regulation of p53, TNF-α and the down-regulation of NFkβ are three major events, which can induce apoptosis in the cells by both intrinsic and extrinsic pathways. The activated p53 and TNF-α in turn activate caspase 3 at downstream. The alteration in the levels of Bax and Bcl-2 (Bax/Bcl-2) at downstream of p53, is also a decisive factor that plays an important role in determining whether cells will undergo apoptosis leading to cell death, or be directed toward the survival pathway. These events push Conium-treated HeLa cells toward apoptosis by altering different cellular proteins' activity.

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