TANG HUMANITAS MEDICINE

Original Article

Homeopathic mother tincture of Conium initiates reactive oxygen species mediated DNA damage and makes HeLa cells prone to apoptosis

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ABSTRACT

Adverse side-effects and lack of scientific validation of some chemotherapeutic agents prevent the use of many traditional medicines claimed to have anti-cancer effects. Ethanolic extract of *Conium maculatum* has long been used in traditional and alternative systems of medicine including homeopathy for the treatment of glandular enlargements, cancerous tumours or hard lumps of testicles, prostate, ovaries, breasts and/ or uterus, particularly in the breast. However, if and how it acts still remains scientifically unknown. This study aims to test if Conium extract (CE), used as mother tincture of Conium in homeopathy, has demonstrable anti-cancer potentials without having much cytotoxicity in normal cells. Cytotoxicity of the drug was tested by conducting MTT assay on both normal (peripheral blood mononuclear cells) and HeLa cells. We also evaluated DNA fragmentation and DNA damage by DAPI and diphenylamine assay. The LDH activity assay was done to evaluate the percentages of apoptosis and necrosis. ROS accumulation also was evaluated to pin-point the actual events of apoptosis. Administration of drug clearly demonstrated its anti-cancer potentials as evidenced by the DNA damage analysis. The ROS activity also increased in case of the CE treated cells. LDH data revealed that the mode of cell death was mainly apoptotic and not necrotic. CE appears to induce apoptosis of cancer cells through ROS mediated pathway, and has negligible cytotoxicity against normal cells.

Keywords *Conium maculatum*, apoptosis, ROS, DNA damage, anticancer potentials, HeLa cells

INTRODUCTION

The cervix carcinoma is responsible for most number of deaths by cancer in female population (Ahemedin et al., 2011). Chemo-prevention against the cervical cancer is not very effective and except for surgical intervention before the metastasis starts, there is hardly any remedial measure that can prove successful to control this cancerous growth (Fulda and Debatin, 2007); therefore, there is an urgent need of remedial drug development strategy to combat this dreadful situation.

Reactive oxygen species (ROS) plays a central part of metabolism in cells, which regulates different cellular functions like cell proliferation, differentiation, self-renewal and removal of unwanted cells through apoptosis and necrosis (Fruehauf and Meyskens, 2007). The normal human cells possess certain level of ROS activity, but the cancer cells contain a higher level of ROS activity due to its higher metabolic rates. If the ROS level could be increased to a further extent that is up to threshold level the cells could undergo apoptosis or necrosis (Lowe and Lin, 2000). This could be a way to control the immortality of cancer cells by drug without damaging the normal cells.

The accumulation of ROS and DNA damage are two inter-dependent events that take place in unison. In other words, the increased level of ROS beyond the threshold level causes increased DNA damage of the cells (Lopez-Lazaro, 2007). If the drug used for the therapy can have the ability to further increase the ROS accumulation, there will automatically be a chance of increase in cellular DNA damage.

The condensation and fragmentation of DNA are major hallmarks of apoptosis. When a drug induces DNA fragmentation, it can have a potent anti-cancer role as well. The changes in cellular morphology (membrane distortion, blebbing) also are the major hallmark of apoptosis (Green, 2011). So, the drug which can induce DNA fragmentation, and cell blebbing, could be considered one with an anti-cancer potential and that can be used to combat cancer.

Homeopathy is a system of medicine founded on a definite law 'Similia Similibus Curantur' which means 'like cures like'. The word Homeopathy is a Greek derivation where 'homeos' means 'similar and pathos means 'suffering'. So Homeopathy may be defined as the therapeutic method of symptom-similarity and ancient ayurvedic texts have on occasions mentioned this law. According to this system, the choice of the medicine is fundamentally based on the principle that the medicine must have the capability of producing most similar symptoms of the disease to be cured in healthy persons (Boericke, 2002; Khuda-Bukhsh, 2003). Therefore, to ameliorate the sufferings of the patients, the use of many types of complementary and alternative medicine (CAM) as supportive cancer care is becoming more popular day by day, and that includes the use of homeopathy. Therefore, the validation of homeopathic remedies by conducting scientifically acceptable protocols is extremely important.

Conium maculatum is derived from the toxic plant hemlock which is native to Europe, particularly Britain, North America, Africa, Chile and certain regions in Asia (http://www.herbs2000.com/homeopathy/conium.htm). Conium is prepared from leaves, roots and flowering stems of hemlock plant by using ethanol as the extracting solvent. In homeopathy, this medicine is generally used to treat glandular enlargements,

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for instance, cancerous tumours or hard lumps of testicles, prostate, ovaries, breasts and/ or uterus, but particularly in glandular enlargement in the breast region. In addition, conium is also prescribed by homeopaths to cure nervous disorders. The homeopathic remedy conium is effective in curing a number of health problems suffered by women. This medicine is particularly useful for aged women who are in their climacteric age (Boericke, 2004). In Conium, eight piperidinic alkaloids have been identified in this species. Two of them, gamma-coniceine and coniine are generally the most abundant and produce plant's acute and chronic toxicity. Conium contains the pyridine alkaloids like coniine, N-methylconiine, conhydrine, pseudoconhydrine and gamma-coniceine, which are the precursors of the other hemlock alkaloids (Schep et al., 2009); these toxic materials may induce the ROS accumulation in the cancer cells, even in lower doses.

In this context, we have tried to evaluate the anticancer activity of conium on Hela cells, for the first time. We deployed several methods like cell viability assay, LDH (Lactate dehydrogenase) enzyme activity, intercellular ROS (reactive oxygen species) activity, morphological study and fluorescence microscopic study of DNA damage to ascertain the effects, if any, of different doses of conium on HeLa cells. As a further confirmatory method to detect apoptosis, DNA fragmentation assay was used. In this study, to provide information on whether the cell death occurs due to apoptosis or necrosis, we also conducted LDH assay.

MATERALS AND METHODS

Cell culture

The Hela cells (NCCS, India) were maintained in the humidified incubator (Thermo, USA) with ambient oxygen and 5% carbon dioxide level. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, USA) supplemented with 10% heat inactivated FBS (fetal bovine serum) (Gibco, NZ) and 1% PSN (penicillin-streptomycin-neomycin antibiotic) (HiMedia, India). Cells were harvested with 1× Trypsin-EDTA (Gibco, Canada) in phosphate buffer saline (SRL, India) and plated at required cell numbers and allowed to adhere for 24 h before treatment. The cells were plated at a density of 2×10^3/well on 96 well tissue culture plates (Tarson, India) for viability experiments, and for 2×10^6 DNA fragmentation assay on 70 mm culture dish (Tarson, India) or 2×10^4 on 6 well plates (Tarson, India) for morphology and fluorescence study, respectively.

MTT/Cell viability assay

In vitro growth inhibition effect of CE on HeLa cells was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) (Sigma, USA) dye absorbance of living cells. The HeLa and PBMC (peripheral blood mononuclear cell) were dispensed in 96 well flat bottom microtiter plates at a density of 2×10^3 cells per well. Exponentially growing cells were exposed to CE at different concentrations for 24 and 48 h. MTT solution was then added to each well and incubated for 4 h at 37°C. To achieve solubilization of the formazan crystals formed in viable cells, media was discarded and 100 µl acidic isopropanol (Merck, India) was added to each well and optical density (O.D.) was measured at 595 nm in an ELISA reader (Thermo scientific, USA). The absorbance correlates linearly to the number of living cells in culture (Chakraborty et al., 2012).

$$\text{Relative percentage viability} = \left(\frac{\text{OD of the drug treated sample}}{\text{OD of the control sample}}\right) \times 100$$

Drug

We used homeopathic mother tincture, *Conium Maculatum*, procured from Boiron Laboratory, Lyon, France. They prepared the drug adopting the guidelines of European Pharmacopoeia 7th (http://www.pharmabooks.com.br/livros/images/livros/Index_7th_Edition_70.pdf) and supplied the drug in 65% ethanol extract. Before the experimental treatment the drug was diluted in the DMEM media. We selected three different doses of CE, namely, D1 (0.15 µg/µl), D2 (0.30 µg/µl), D3 (0.45 µg/µl) for all the experiments. 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 only the positive control for comparison of results.

LDH assay

LDH activity was assessed using a standardized kinetic determination kit (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℃ for 2 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). 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) (Mandal et al., 2010). The percentages of apoptotic and necrotic cell deaths were calculated as follows:

$$\text{Apoptosis percentage} = \left(\frac{\text{LDHp}}{\text{LPHp} + \text{LDHi} + \text{LDHe}}\right) \times 100$$

$$\text{Necrosis percentage} = \left(\frac{\text{LDHe}}{\text{LPHp} + \text{LDHi} + \text{LDHe}}\right) \times 100$$

Cell morphology

HeLa cells were plated in 6 well culture plates (2×10^4 cells/well) in DMEM supplemented with 10% FBS. Following incubation with CE for desired period, the cells were observed with an inverted phase contrast microscope [Zeiss, Germany] and photographs were taken.

Fluorescence microscopic study of DAPI staining

In order to detect whether CE induced nuclear chromatin condensation, staining with DAPI was performed. Control and treated cells were stained with 10 µg/ml of DAPI (4',6' Diamidino-2-phenylindole dihydrochloride) (Sigma, USA), visualized under fluorescence microscope and photograph taken with 20× magnification and photographs were taken using Moticam and analysed by Motic Image software.

Fluorescence microscopic study of DCHFDA staining

In order to detect whether CE induced ROS accumulation, staining with DCHFDA (2',7'-Dichloro Dihydro Fluorescein Diacetate) was performed. Control and treated cells were stained with 10 µg/ml of DCHFDA (Sigma, USA), visualized under fluorescence microscope and photograph taken with 10×

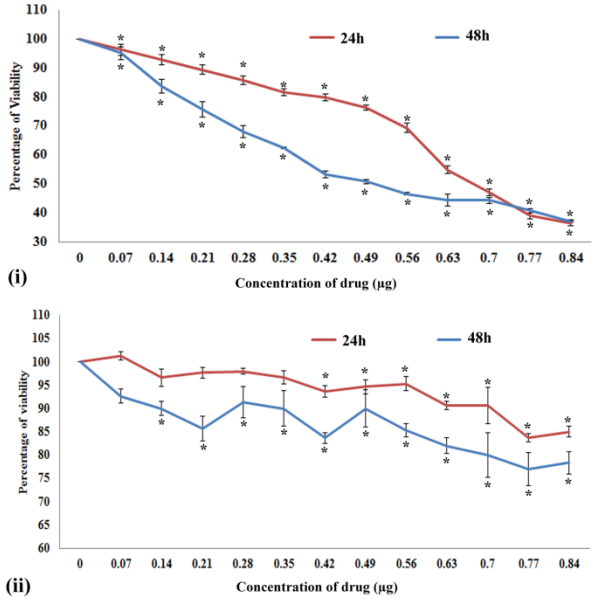

Fig. 1. CE induced HeLa cells and PBMC viability. Effect of CE on HeLa cells (i) and on PBMC (ii) incubated for 24 h and 48 h at different concentrations (0.07 - 0.84 µg/µl) after the adherence of the cells. The cell viability was then determined by MTT assay. The results were expressed as Mean ± SE (N = 3) [Significance, $^{*}p < 0.05$ vs. normal control group].

magnification and photographs were taken using Moticam and analysed by Motic Image software.

Quantification of DNA fragmentation

1 ml of cell suspension (2×10^6) was harvested at 200g. The supernatant (S) and pellet (B) both was dissolved in Tris-buffer-Triton-X (TTE) (SRL, India) solution with vigorous vortexing. The suspension (B) was then centrifuged at 20,000 g for 10 min at 4°C (REMI, India). The supernatant was

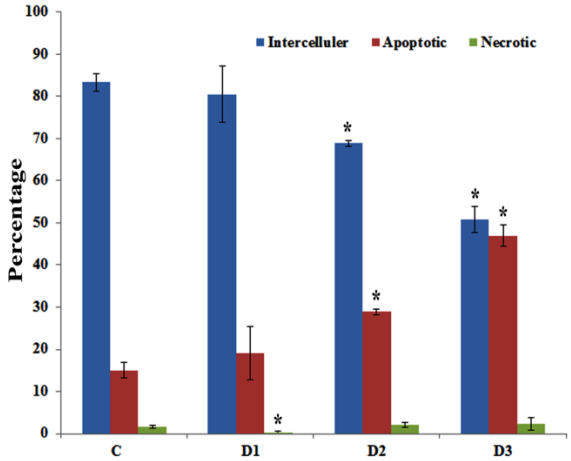

Fig. 2. LDH assay: Apoptosis and necrotic percentage. Apoptosis and necrosis percentage was determined at different drug concentrations by LDH activity assay. The results are expressed as Mean ± SE (N = 3) [Significance, $^{*}p < 0.05$ vs. normal control group]. In the bar diagram, blue colour represents the live cells, red represents apoptotic and green colour represents the necrotic mode of cell death.

transferred to new tube (T). 25% trichloro acetic acid (TCA) (Loba Chemi, India) was mixed vigorously with S, B and T. The precipitation was allowed for overnight at 4°C. After removing the supernatant, DNA was hydrolysed by adding 160 µl of 5% TCA and heating 15 min at 90°C in a heating block. Then to each tube, freshly prepared DPA (diphenylamine) (SRL (India) solution was added and the mixture vortexed well and kept for colour development for overnight. 200 µl coloured solution was transferred to 96 well plate avoiding the dark particles (Ioannou and Chen, 1996). The OD was taken at 600nm in ELISA reader using a blank (160 µl of 5% TCA and 320 µl DPA alone).

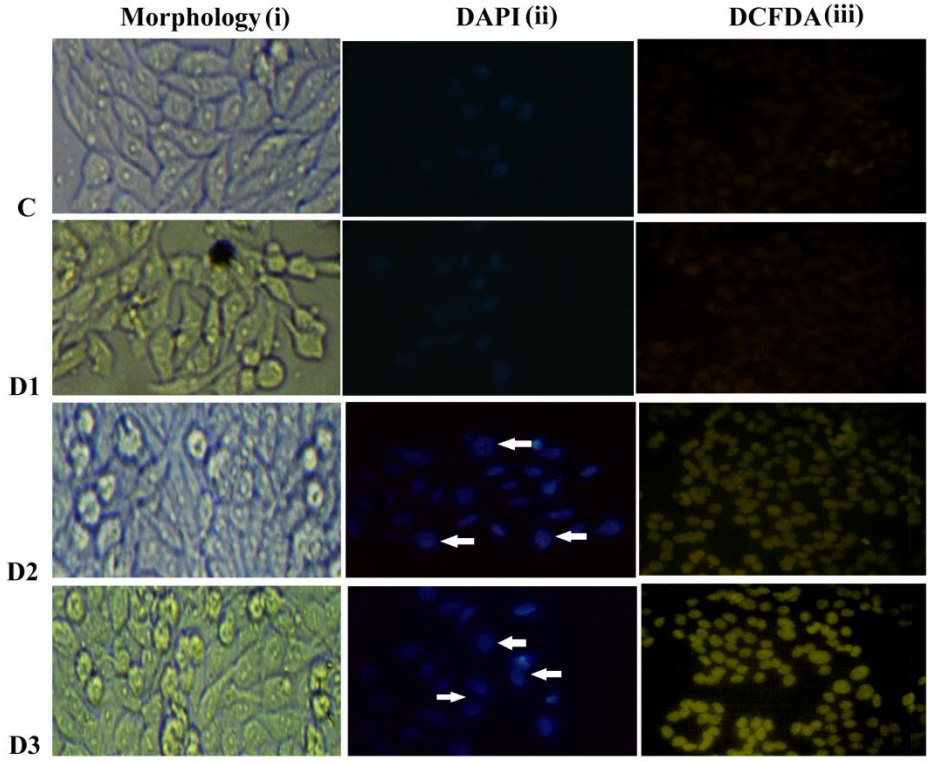

Fig. 3. Microscopic studies. The cells were treated with D1 (0.15 µg/µl), D2 (0.30 µg/µl), D3 (0.45 µg/µl) of drug to check the different aspects of the anticancer drugs, (i) Morphological study: The morphology of the HeLa cells changed with the increasing drug doses with respect to the control set. The rounding of the cell structure and the morphology distortion generally indicated the drug's effect on the cancer cells. (ii) DAPI staining: DAPI can bind to the fragmented DNA. The more amount of fragmentation gave more fluorescence intensity. The fragmentation was observed to be in the highest range at the highest drug dose. (iii) DCHFDA staining: more amounts of ROS were generated in the higher drug dose treated cells than the control set.

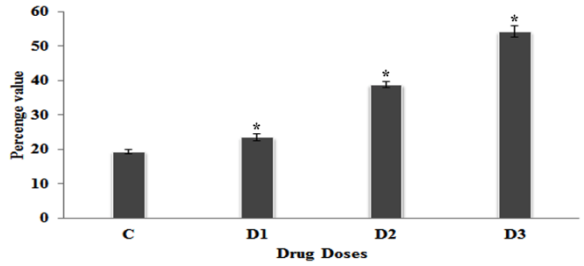

Fig. 4. DNA fragmentation assay. Effect of CE on the DNA through DNA fragmentation assay by diphenylamine (DPA). The results were expressed as Mean ± SE (N = 3) [Significance, $^{*}p < 0.05$ vs. normal control group].

$$\text{Percentage of fragmentation} = \frac{(S + T)}{(S + T + B)} \times 100$$

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

Evaluation of cell viability

Results of MTT assay revealed that the cell viability was gradually decreased from minimal drug concentration to the higher ones at both 24 and 48 h. The percentages of viability were: 96.21 ± 1.98 at the dose 0.07 µg/µl to 36.48 ± 0.99 at the dose 0.84 µg/µl of drug at 24 h; for the 48 h fixation time point, the percentages of cell viability were: 95.11 ± 2.19 at 0.07 µg/µl to 36.93 ± 0.27 at 0.84 µg/µl of drug. The 50% cell death occurred at 0.72 ± 0.01 µg/µl at 24 h and 0.57 ± 0.01 at 48 h of drug employment (Fig. 1i). The cell cytotoxicity on normal PBMC was also measured in the mentioned dose of CE which showed 85 ± 1.15 and 78.33 ± 2.4 at highest drug dose for 24 and 48 h, respectively (Fig. 1ii) to PBMC.

Determination of apoptosis and necrosis percentages

The percentage value of cell death by apoptosis and necrosis was determined by LDH activity assay. After the drug treatment the apoptotic percentages were increased from 15.04 ± 1.82 (C) to 19.12 ± 6.34, 28.92 ± 0.68 and 46.96 ± 2.47 in case of D1, D2 and D3 doses, respectively. The necrotic percentages were relatively lower in the drug treated cells than in the control set, and they were 1.62 ± 0.28, 0.35 ± 0.35, 2.15 ± 0.54 and 2.26 ± 1.49 for C, D1, D2 and D3, respectively (Fig. 2).

Microscopic studies

Cellular morphology

The morphological distortion and the cell blebbing were observed in the drug treated cells. The cellular distortion was found to be most frequent in case of higher doses (Fig. 3i).

DAPI staining

The fragmented DNA was observed in the drug treated cells than in the control ones. The DNA breakage mostly occurred in case of the highest drug dose (Fig. 3ii) used.

DCHFDA staining

The amount of ROS accumulation was higher in case of the higher doses. The blue colour intensity was gradually increased in the higher drug doses. The D3 had the most ROS accumulation and has the highest amount of florescence (Fig. 3iii).

Analysis of DNA fragmentation assay

The amount of fragmented DNA was increased with the drug treatment. The control set contained 19.34 ± 0.57% of fragmented DNA and upon the drug treatment, the fragmented percentages were 23.49 ± 0.98, 38.84 ± 0.98 and 54.27 ± 1.74 for D1, D2 and D3, respectively (Fig. 4).

DISCUSSION

Cervix cancer is a major health problem in females and chemotherapy or radio therapy is often found to be ineffective after a certain period of treatment, because the cancer cells become unresponsive to the therapy (Massague, 2004). Additionally, unwanted side-effects and/or the toxicity of the conventional (orthodox) drugs in normal tissues and normal cells often make the problem complicated. We observed that CE reduced the viability of the cancerous cells but not of the normal cells and induced apoptotic condition to the cancer infected cells.

Initiation of necrotic cell death is another problem with the conventional anti-cancer drugs due to their higher cytotoxic nature, so in the drug designing, this factor should be taken care (Lemasters et al., 1998) of. A potent complete anti-cancer drug can only initiate apoptosis to the cancer cells but not necrosis (Kim et al., 2003). The CE used in this study initiated apoptosis to the HeLa cells with a negligible necrotic cell death. The LDH activity assay also supported this nature of the drug.

The initiation of cellular morphology change and DNA fragmentation are two major hallmarks of cancer (Green, 2011). The apoptotic cells' chromosome gets condensed and fragmented. The drug induced both the cellular phenomena in the HeLa cells.

ROS is the central component and can control different cellular behaviours. The cancer cells possess a higher level of ROS due to their high metabolic activity. But, if a drug can increase ROS level of cells to a further extent, raising it beyond the threshold level, then they can push the cells more strongly to the apoptosis (Lopez-Lazaro, 2007). In this study, the ROS level was found to be increased by the drug induction. The accumulation ROS has the capacity to induce DNA damage of the cells, and this DNA damage can lead them to death. The DCHFDA and DAPI staining also supports the cellular ROS accumulation and DNA damage upon drug treatment in the HeLa cells.

In conclusion, we can state that, the CE has the ability to accumulate reactive oxygen species in the cancer cells and thus leads the cells to apoptotic pathway through the cytotoxic effects, but it has less cytotoxic effect to the normal cells.

Thus, the critical analysis of overall data would convincingly demonstrate the anti-cancer potentials of CE and the information would be important in designing specific drug for chemo-therapeutics, apart from validating its use in homeopathy and CAM practices as an anti-cancer drug.

AUTHOR DISCLOSURE STATEMENT

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CONFLICT OF INTEREST

No authors/co-authors have any competing interest to declare.

REFERENCES

Ahemedin J, Bray F, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.

Boericke W. Pocket manual of homeopathic material medica & repertory: Comprising of the Characteristic and Guiding Symptoms of All Remedies (clinical and Pahtogenetic [sic]) Including Indian Drugs. reprint ed. (New Delhi, India: B. Jain Publishers), 2002.

Chakraborty D, Mukherjee A, Sikdar S, Paul A, Ghosh S, Khuda-Bukhsh AR. [6]-Gingerol isolated from ginger attenuates sodium arsenite induced oxidative stress and plays a corrective role in improving insulin signalling in mice. Toxicol Lett. 2012;210:34-43.

Fruehauf JP, Meyskens Jr FL. Reactive oxygen species: a breath of life or death?. Clin Cancer Res. 2007;13:789-794.

Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene. 2006;25:4798-4811.

Green JA. Clinical trials in cancer. Br J Cancer. 2011;104:1521-1522.

Ioannou YA, Chen FW. Quantitation of DNA fragmentation in apoptosis. Nucleic Acids Res. 1996;24:992-993.

Khuda-Bukhsh AR. Towards understanding molecular mechanisms of action of homeopathic drugs: An overview. Mol Cell Biochem. 2003;253:339-345.

Kim JS, He L, Lemasters JJ. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem Biophy Res Commun. 2003;304:463-470.

Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophy Acta. 1998;1366:177-196.

Lopez-Lazaro M. Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. Cancer Lett. 2007;252:1-8.

Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis. 2000;21:485-495.

Mandal SK, Biswas R, Bhattacharyya SS, Paul S, Dutta S, Pathak S, Khuda-Bukhsh AR. Lycopodine from Licopodium clavatum extract inhibits proliferation on HeLa cells through induction of apoptosis via caspase-3 activation. Eur J Pharmacol. 2010;626:115-122.

Massague J. G1 cell-cycle control and cancer. Nature. 2004;432:298-306.

Schep LJ, Slaughter RJ, Beasley DM. Nicotinic plant poisoning. Clin Toxicol. 2009;47:771-781.