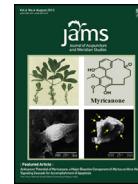


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RESEARCH ARTICLE

The Potentized Homeopathic Drug, *Lycopodium clavatum* (5C and 15C) Has Anti-cancer Effect on HeLa Cells *In Vitro*.

Asmita Samadder ¹, Sreemanti Das ¹, Jayeeta Das ¹, Avijit Paul ¹,
Naoual Boujedaini ², Anisur Rahman Khuda-Bukhsh ^{1,*}

¹ Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani, West Bengal, India

² Boiron Laboratory, Lyon, France

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signal proteins

Abstract

Cancer is a disease that needs a multi-faceted approach from different systems of medicine. The purpose of this study was to evaluate whether homeopathically-potentized ultra-high dilutions of *Lycopodium Clavatum* (LC-5C and LC-15C, respectively) have any anti-cancer effects on HeLa cells. Cells were exposed to either LC-5C (diluted below Avogadro's limit, i.e., 10^{-10}) or LC-15C (diluted beyond Avogadro's limit, i.e., 10^{-30}) (drug-treated) or to 30% succussed ethanol ("vehicle" of the drug). The drug-induced modulation in the percent cell viability, the onset of apoptosis, and changes in the expressions of Bax, Bcl2, caspase 3, and Apaf proteins in inter-nucleosomal DNA, in mitochondrial membrane potentials and in the release of cytochrome-c were analyzed by utilizing different experimental protocols. Results revealed that administration of LC-5C and LC-15C had little or no cytotoxic effect in normal peripheral blood mononuclear cells, but caused considerable cell death through apoptosis in cancer (HeLa) cells, which was evident from the induction of DNA fragmentation, the increases in the expressions of protein and mRNA of caspase 3 and Bax, and the decreases in the expressions of Bcl2 and Apaf and in the release of cytochrome-c. Thus, the highly-diluted, dynamized homeopathic remedies LC-5C and LC-15C demonstrated their capabilities to induce apoptosis in cancer cells, signifying their possible use as supportive medicines in cancer therapy.

* Corresponding author. Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India.
E-mail: prof_arkb@yahoo.co.in, khudabukhsh_48@rediffmail.com (A.R. Khuda-Bukhsh).

1. Introduction

Cervical cancer is the third most common type of cancer in women worldwide. It carries few or no obvious symptoms until it has progressed to an advanced stage. Therefore, the treatment usually consists of chemotherapy and/or radiotherapy [1]. These orthodox modes of treatment are often painful and are associated with considerable side effects. Further, people often look for other modes of treatment that have relatively fewer or no side effects and can complement the orthodox treatment.

Homeopathy is one such arm of complementary and alternative therapy that causes the patient no pain and has no or negligible side effects with use [2]. Homeopathic medicines are available at affordable prices and are therefore becoming increasingly popular in many developing countries. In spite of the growing popularity of homeopathy, concerns still remain in respect of its experimental validation and mechanism of action. In particular, questions are often raised about the rationale behind using ultrahigh dilutions of the remedies (exceeding Avogadro's limit), which are not expected to contain even a single molecule of the original drug substance as a result of the repeated dilutions [3]. Therefore, validation through well-designed experiments is greatly warranted to convince both skeptics and believers.

To understand the concept of "potentization" in the centesimal scale (C), the homeopathic procedure of dilution needs to be explained. First, 1 mL of the "mother tincture", the crude ethanolic extract of *Lycopodium clavatum*, is diluted with 30–90% ethanol (mostly 90% and 70%, but in some cases 30% is also used) and given 10 uniform jerks to make the potency 1C. A volume of 1 mL of potency 1C is then diluted with 99 mL of ethanol and given 10 jerks or succussions to make the potency 2C, and so on [4,5]. Thus, there may be a physical presence of some original drug molecules at potency 5C, but it is highly improbable that potency 15C will contain even a single original molecule of the drug because the dilution has gone beyond Avogadro's limit.

Homeopathic practitioners use mother tinctures, as well as potentized remedies diluted to both below and above Avogadro's limit, depending on the patient's condition, and claim to find all remedies effective against the disease symptoms for a given condition. Therefore, in this study, we chose two potencies, 5C (below Avogadro's limit) and 15C (above Avogadro's limit), to test primarily whether the diluted potencies could still show efficacy in leading the otherwise immortal cancer cells HeLa to apoptosis (cell death) in culture conditions. Incidentally, many earlier reports have recorded efficacy of both the lower and the higher potencies of homeopathic remedies [6–9].

2. Materials and methods

2.1. Reagents

MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide], secondary antibodies, and all other chemicals

were purchased from Sigma-Aldrich Inc. (St-Louis, MO, USA). The primary antibodies (Bcl2 associated X (Bax), B cell lymphoma 2 (Bcl2), caspase 3, Apoptotic protease activating factor 1 (Apaf1), and cytochrome C) were procured from Santa Cruz, California, U.S.A. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and penicillin, streptomycin, and neomycin (PSN) antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). All organic solvents used were of high-performance liquid chromatography grade.

2.2. Cell culture

The human cervical cancer cell line HeLa was procured from National Centre for Cell Science (NCCS, Pune, India). The cells were cultured at 5×10^5 cells/mL in DMEM supplemented with 10% fetal bovine serum and 1% PSN antibiotic at 37°C in 5% CO₂ for experimental purposes.

2.3. Assessment of viable cells

The viability of the HeLa cells was evaluated by the MTT assay [10] after exposure to LC-5C and LC-15C. The cells were incubated in 96-well microplates for 24 hours with and without the drugs. The intracellular formazan crystals formed were solubilized with dimethyl sulfoxide, and the absorbance of the solution was measured at 595 nm using a microplate reader (Multiskan ELISA; Thermo Scientific, Brookfield, WI, United States). The cell survival percentage on MTT assay was calculated using the following equation: percentage of survival = (mean experimental absorbance / mean control absorbance) × 100.

2.4. Use of negative control, placebo control, and selection of dose of the drugs

Untreated HeLa cells were used as a negative control, while the vehicle-treated (placebo) treated samples were used as a positive control. Additionally, a widely used chemotherapeutic drug, cisplatin [11], from the orthodox regimen, was also used to make a basic comparison of the cancer cell-killing efficiency of the two tested drugs from two different systems, by determining the percentage survivability using the MTT assay. Because the results of the primary MTT assay revealed that cisplatin and both potencies of the homeopathic drug (LC-5C and LC-15C) showed an almost similar apoptotic effect on the HeLa cervical cancer cells, we did not deem it necessary to proceed further with other parameters of the study using this control as our primary objective was to evaluate only the anticancer potential of the potentized homeopathic drug.

Two different doses of LC-5C and LC-15C were selected for further experiments based on their respective LD₅₀ values. The doses that were selected for LC-5C and LC-15C were: 5 µL/100 µL media [LC-5C(I)], 6 µL/100 µL media [LC-5C(II)], 7 µL/100 µL media [LC-15C(I)], and 8 µL/100 µL media [LC-15C(II)], respectively. Normal peripheral blood mononuclear cells (PBMCs) were also cultured and exposed to LC-5C and LC-15C, in a concentration determined from MTT assay, to test their cytotoxic effect, if any [12].

2.5. Lactate dehydrogenase assay

The cellular membrane integrity of HeLa cells was checked at 24 hours of incubation with LC-5C and LC-15C using the activity of lactate dehydrogenase (LDH) released from the cytosol of damaged cells into the supernatant; this was measured using an LDH determination kit (LDH1144; Crest Biosystems, Goa, India). The floating cells were collected from the culture medium by centrifugation (1000 g) at 4°C for 5 minutes, and the LDH content of the pellets (LDHp) was used as an index of apoptotic cell death [11]. The LDH released to 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 death were calculated as follows:

$$\text{Apoptotic percentage} = [\text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe})] \times 100$$

$$\text{Necrotic percentage} = [\text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe})] \times 100$$

2.6. Fluorescence study by Hoechst 33342 and propidium iodide staining

The extent of apoptosis in the HeLa cells was assessed by Hoechst 33342 and propidium iodide (PI) staining (1 µg/mL), and the fluorescence intensity was examined using a fluorescence microscope (Leica, Wetzlar, Germany) [10,13].

2.7. Assessment of mitochondrial membrane potential by rhodamine 123

The changes in the mitochondrial membrane potential (MMP) of the cells were determined using a fluorescent probe, rhodamine 123 [14]. Briefly, the HeLa cells were incubated using the two selected doses, LC-5C and LC-15C, for 24 hours. The cells were then washed in phosphate-buffered saline (PBS), and 5 µL of rhodamine 123 (1 µM) was added to the cells (2×10^5). The cells were then incubated for 15 minutes. Next, the cells were washed with PBS and observed under a fluorescence microscope (Leica).

2.8. Double labeling of cells with Annexin V–fluorescein isothiocyanate and PI

Measures of 1×10^6 cells per sample were placed into small centrifuge tubes. The cells were spun at 1200 g for 5 minutes, the supernatant was discarded, and the pellets were washed with 500 µL binding buffer. Then cells were harvested at 1200 g for 5 minutes, and the cell pellets were resuspended in 80 µL binding buffer. Annexin V–fluorescein isothiocyanate (FITC) 10 µL and 10 µL of PI labeling solution were added, and the cell suspension was incubated for 15 minutes at room temperature in the dark. The solution was then ready for analysis by flow cytometry [15].

2.9. Estimation of DNA damage

The genomic DNA was extracted from HeLa cells by a standardized phenol–chloroform method, and DNA gel electrophoresis was performed in 1% agarose gel [16].

2.10. Expression of proteins by indirect enzyme-linked immunosorbent assay study

Enzyme-linked immunosorbent assay (ELISA) studies were carried out using a nuclear extract of the cells to quantify the expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Bax, Bcl2, Apaf, cytochrome C, and caspase 3 proteins according to a standard protocol [14].

2.11. Statistical analysis

Data were presented as means ± standard error (SE). Statistical analysis was performed using the Student paired *t* test analysis and one-way analysis of variance (ANOVA); * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ were considered significant.

3. Results

3.1. Proliferative assay: by MTT

The proliferation of HeLa cells decreased with an increase in concentration of LC-5C and LC-15C. The percentage of cell viability is shown in Fig. 1A. From the curve of the HeLa cells (5×10^5 cells/mL), it can be clearly observed that 5 µL/100 µL media of LC-5C and 7 µL/100 µL media of LC-15C decreased cell viability to 50.94% and 50.98%, respectively, at 24 hours. This trend of a decrease in the percentage cell viability of the HeLa cells was found to be similar to that of the chemotherapeutic drug cisplatin (50.3% at 24 hours of incubation and at a 20 µM dose; Fig. 1B). This suggests that both our drugs of interest, LC-5C and LC-15C, could be further evaluated for their anticancer potency. The percentage cell cytotoxicity of the drugs was also checked on normal PBMCs, resulting in minimal cytotoxic effect (Fig. 1C).

3.2. Assessment of apoptotic and necrotic cells by LDH assay

The ratio of LDH released from apoptotic and necrotic cells showed a significant increase in the ratio of apoptotic cells compared to necrotic cells (Fig. 1D). The ratio increased in a dose-dependent manner.

3.3. PI and Hoechst 33258 staining

HeLa cells treated with LC-5C and LC-15C stained positively with PI (Fig. 2A) and Hoechst 33258 (Fig. 2B), with visible chromatin condensation compared to untreated control cells.

The fluorescence-activated cell sorting (FACS) data to assess apoptosis using Annexin V–FITC staining (Fig. 3A) indicated a greater apoptotic potential of LC-5C than LC-

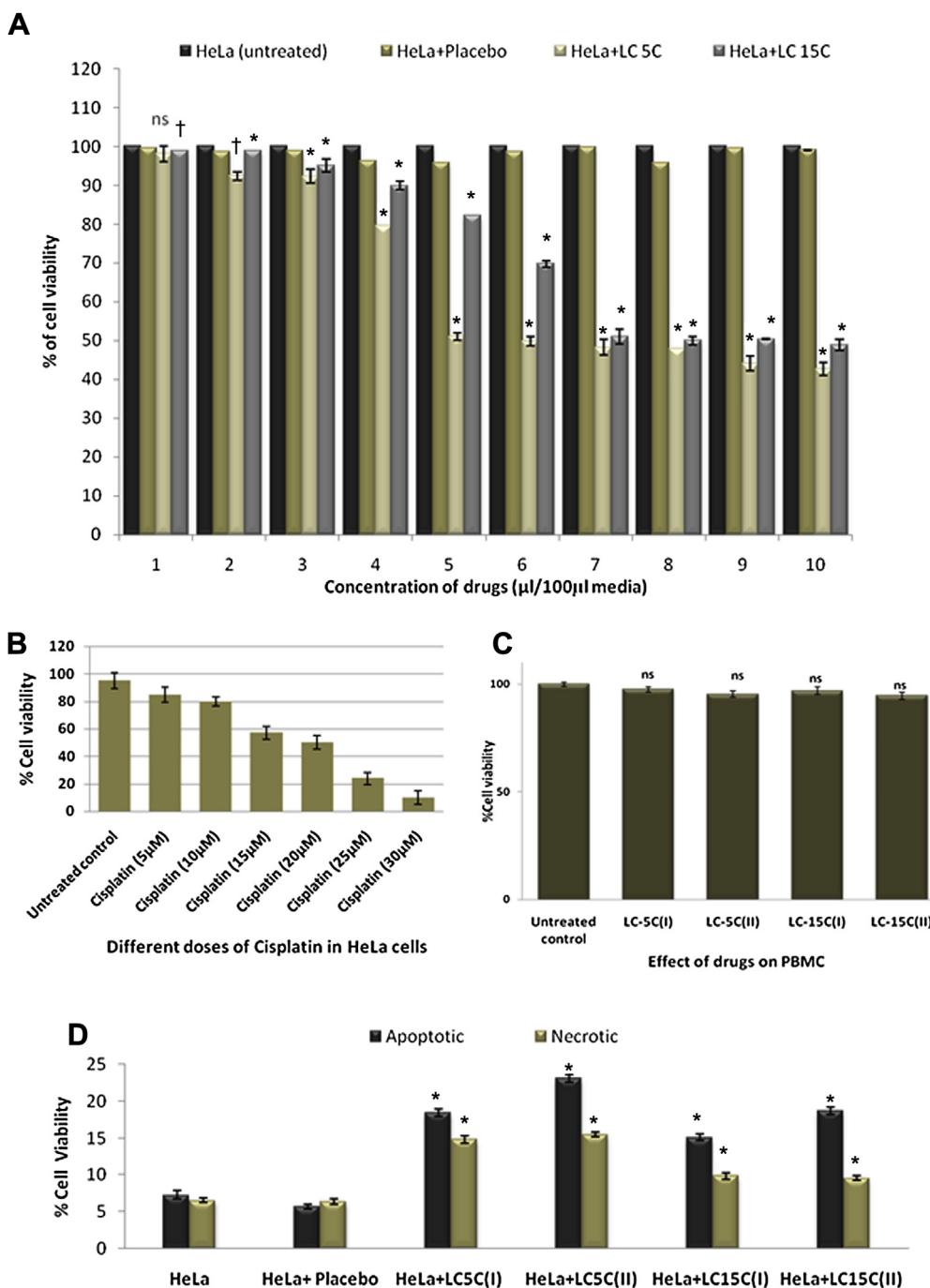


Figure 1 (A) Assessment of cell viability of HeLa cells before and after treatment with the drugs. (B) Assessment of cell viability of HeLa cells before and after treatment with cisplatin, a potent anticancer drug. (C) Assessment of cell viability of peripheral blood mononuclear cells (PBMCs) before and after treatment with the drugs. (D) Assessment of lactate dehydrogenase (LDH) activity of HeLa cells before and after treatment with the drugs. * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ versus placebo (alcohol) treated series.

15C, which also showed considerable apoptotic activity at the higher dose.

3.4. Analysis of MMP

Compared to the control, the MMP of the drug-treated HeLa cells showed a marked decrease in the intensity of the

greenish stain, indicating an effective depolarization of MMP (Fig. 3B).

3.5. Assessment of damage of DNA

DNA gel electrophoresis data showed that the LC-5C- and LC-15C-treated groups showed a clear DNA smearing

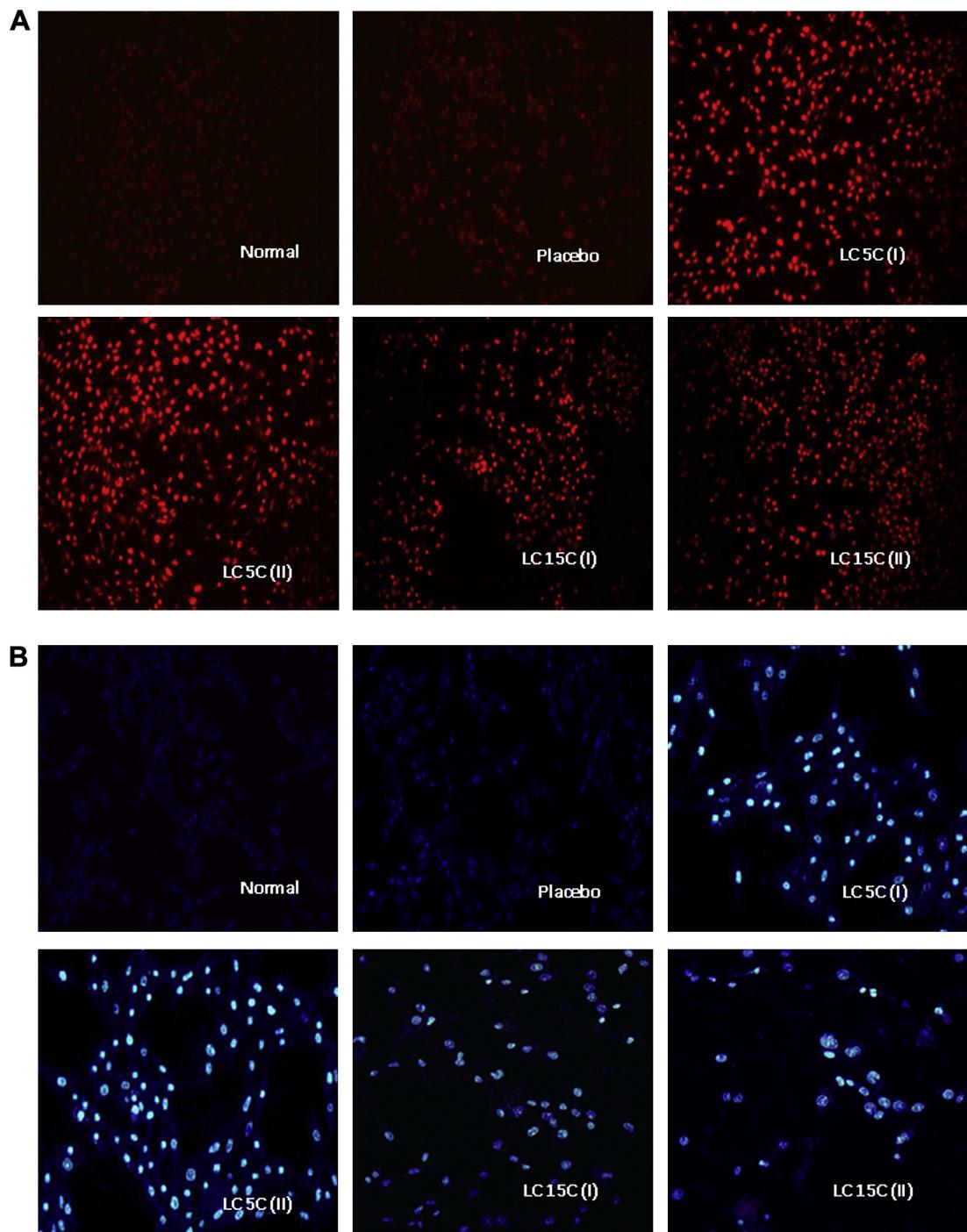


Figure 2 (A) Apoptotic assay of HeLa cells by propidium iodide (PI) staining before and after treatment with the drugs. (B) Apoptotic assay of HeLa cells by Hoechst 33342 staining before and after treatment with the drugs. * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ versus placebo (alcohol) treated series.

when compared to the single band of the control cells (Fig. 4A).

3.6. Indirect ELISA study

A significant increase in the expressions of Bax, Apaf, cytochrome C, and caspase 3 and a decrease in the expression of Bcl2 were observed in the LC-5C- and LC-15C-treated groups compared to untreated cells.

4. Discussion

The hallmark feature of cancer cells that makes them virtually immortal and unconquerable is their capability for uncontrolled cell proliferation and ability to ignore signals relating to apoptosis or cell death. Any external agent or drug that can prevent, interfere with, or slow down the process of proliferation or lead the cells to cell death by apoptosis or necrosis can be considered as being

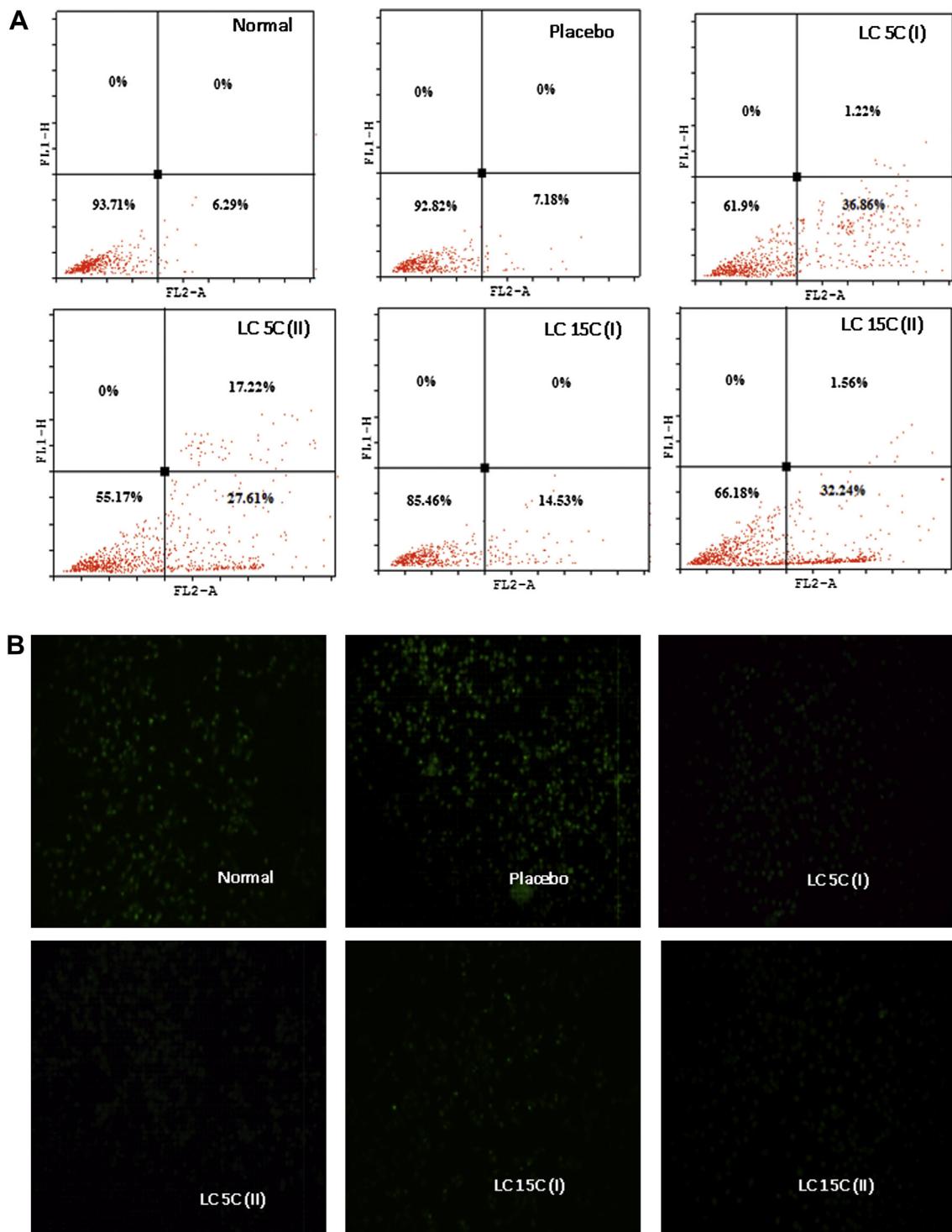


Figure 3 (A) Assessment of apoptosis by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) before and after treatment with the drugs. (B) Qualitative analysis of mitochondrial membrane potential (MMP) in different sets of experimental cells. * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ versus placebo (alcohol) treated series.

of substantial importance, particularly if it is in a low dose and devoid of any adverse side effects. The present findings support the theory that both LC-5C and LC-15 induced a considerable percentage of cells to undergo apoptosis, as revealed from the results of the different study parameters. Therefore, the efficacy of both

potencies of LC often used by homeopathic practitioners in the treatment of certain disease conditions (symptoms) including cancer, which would call for this remedy, can at least be vindicated because the efficacy of these remedies was demonstrated in the HeLa cancer cells. The findings of the present study also support the claims made

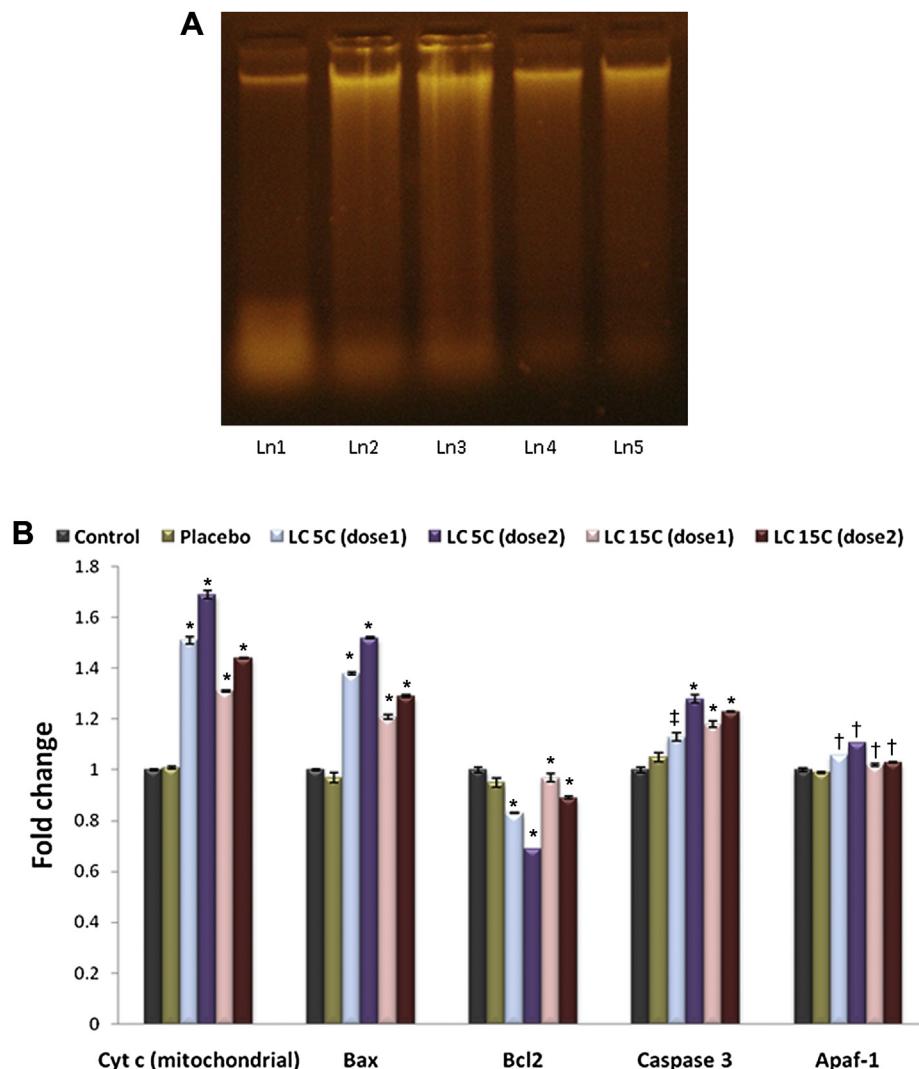


Figure 4 (A) Estimation of DNA damage by gel electrophoresis of genomic DNA. (B) Quantitative expression (fold change) of different proteins by indirect enzyme-linked immunosorbent assay (ELISA). * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ versus placebo (alcohol) treated series.

by several other studies previously carried out in similar areas [8,9].

The possible ways in which both LC-5C and LC-15C could act were probably via the apoptotic signaling pathways. This was evident from the fact that there were definite changes in the parameters associated with apoptosis, such as loss of percentage of viable cells, nuclear condensation, laddering of genomic DNA, significant increase in LDH release, expression of apoptotic genes, and so on, after administration of LC-5C and LC-15C to HeLa cells, while these favorable modulations were lacking in the placebo-treated cells.

Mitochondria are known to be sensitive to changes in the redox state of cells [17]. Maintenance of mitochondrial membrane integrity is a dynamic process. Therefore, the release of cytochrome C proteins into the cytosol might actively contribute to the process of apoptosis [18]. Because apoptosis is highly dependent on the release of certain mitochondrial enzymes, which is in turn associated with mitochondrial membrane depolarization, we also

studied this aspect of mitochondrial transmembrane potential in view of its implications for apoptosis. Both drugs showed positive modulations in this respect, thereby implying that the drugs led the cancer cells to undergo apoptosis via the release of cytochrome C into the cytosol.

There was clear evidence of upregulation and downregulation in the expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl2) genes, respectively, which provides an insight into the fact that these drugs could initiate a favorable change in the signal transduction mechanism, which is involved in triggering and regulating the mechanism of apoptosis in an orderly and sequential manner. Activation of the caspase cascade is a well-known molecular mechanism involved in the induction of apoptosis, and caspase 3 is the key factor in the execution of apoptosis [19]. There was an overexpression of caspase 3 in the HeLa cells in the LC-5C- and LC-15C-treated groups at 24 hours, whereas the placebo could not elicit such an effect. The evidence that indicates the involvement of the phenomenon of apoptosis as the main process of cancer cell

destruction could be visualized through an increase in the percentage of cell damage, DNA damage, changes in membrane potential, and activation of the caspase cascade proteins. Although it is very difficult to explain why and how this highly diluted drug could execute the relevant modulations, there were certain clues suggesting that the potentized homeopathic drugs were possibly triggering action-specific genes that could have a regulatory influence on the expression of other specific genes responsible for bringing about the correct functioning of the recovery process, which had gone astray in the diseased state.

The effect of dilution and succussion has been claimed to be of enormous importance in the process of dynamization of potentized homeopathic remedies. When the drug was diluted to 12C or above, it crossed Avogadro's limit, meaning that the existence of even one molecule of the original drug substance became highly improbable—this is the controversy that keeps many from believing in the efficacy of such an ultrahigh dilution. Thus, the results of well-designed experiments using the homeopathically potentized diluted drugs and scientifically acceptable parameters of study are highly significant, even more so because of the fact that the same dilutions of the succussed ethanol (placebo) without the initial drug substance failed to elicit similar responses at the molecular level [2,3,20].

Therefore, the results of the present investigation actually vindicated the hypothesis long advocated by Khuda-Bukhsh and his coworkers [4,5,20,21] and collaborators [22] that one major way by which a potentized homeopathic drug would act is by regulating the expression of relevant genes, presumably by epigenetic modification. However, further work will be necessary to clearly depict the molecular pathway of action of ultrahigh diluted homeopathic remedies.

Conflict of interest

The authors declare that there are no conflict of interest.

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