# Homeopathy

## The Journal of the Faculty of Homeopathy

Volume 106 • Issue 4 November 2017 Pages 203-213

- Homeopathic *S. jambolanum* and *C. indica* in diabetic rats
- Antilithic potential of *B. vulgaris* in rats
- Stimulation of maize germination and growth
- Homeopathy during 2009 A/H1N1 influenza pandemic in India
- Homeopathy in Chikungunya
- Homeopathy in rural Australian primary care
- HPT of Hepatitis C
- Rebound effects of immunomodulatory drugs
- Phenomenology and homeopathy

ELSEVIER

Homeopathy (2017) 106, 203–213

https://doi.org/10.1016/j.homp.2017.09.001, available online at http://www.sciencedirect.com

ORIGINAL PAPER

# Therapeutic potential of HIV nosode 30c as evaluated in A549 lung cancer cells

Anisur Rahman Khuda-Bukhsh[1,*,a], Jesmin Mondal[1] and Rajesh Shah[2]

[1]*Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, India*
[2]*Life Force, 411 Krushal Commercial Complex, G.M. Road, Chembur, Mumbai 400 089, India*

***Objectives:*** **To examine if HIV nosode in 30c dilution (HIV 30c) has therapeutic potential against lung cancer cells (A549) as compared to WRL-68 normal cells and to elucidate its possible molecular mechanism of action on DNA replication and apoptosis.**
***Methods:*** **Effects of HIV 30c were thoroughly tested for its possible anticancer potential on A549 cells (lung cancer); WRL-68 normal liver cells served as control. Three doses, one at LD50 and two below LD-50, were used. Proliferation, migration and senescence assays were made and generation of reactive oxygen species (ROS) studied by routine techniques. The ability of HIV 30c to induce apoptosis in A549 cells and its possible signalling pathway were determined using immunoblots of relevant signal proteins and confocal microscopy, including studies on telomerase reverse transcriptase (TERT) and topoisomerase II (Top II) activities, intimately associated with cell division and DNA replication.**
***Results:*** **HIV 30c prevented cancer cell proliferation and migration, induced pre-mature senescence, enhanced pro-apoptotic signal proteins like p53, bax, cytochrome c, caspase-3 and inhibited anti-apoptotic signal proteins Bcl2, TERT and Top II, changed mitochondrial membrane potential and caused externalization of phosphatidyl serine. Thus, it induced apoptosis as also evidenced from increase in cells with distorted membrane morphology, nuclear condensation, DNA fragmentation, and ROS, typical of apoptosis in progress.**
***Conclusion:*** **HIV 30c nosode has therapeutic potential for inducing cytotoxic effects on A549 cells as manifested by changes in nuclear condensation, DNA fragmentation, ROS generation and MMP, and for its inhibitory action on cell proliferation, cell migration, expression of telomerase reverse transcriptase and Top II genes, and increasing expression of pro-apoptotic genes.** *Homeopathy* (2017) **106**, 203–213.

**Keywords:** HIV nosode; Anticancer potential; A549 cells; Telomerase reverse transcriptase; Topoisomerase-II

## Introduction

Infection by human immunodeficiency virus (HIV) initially produces a brief period of influenza-like illness, but then the virus usually replicates a single stranded DNA by reverse transcriptase activity and gets incorporated into the host genome without producing any notable symptom for a variable period of time.[1] The gene product from the host genome then begins to cause a progressive loss of CD4+ T-cells, leading to almost total dysregulation of the immune system, and the victim now becomes extremely susceptible to common infections, like tuberculosis, and other opportunistic infections and tumours. The late symptoms of acquired immune deficiency syndrome (AIDS) often get complicated by a particular infection of the lung known as "pneumocystis pneumonia", associated with severe weight loss, skin lesions

*Correspondence: Present address: Anisur Rahman Khuda-Bukhsh, B-2/325, Kalyani, Husn Ara Manzil, Kalyani 741235, India.
E-mail: prof_arkb@yahoo.co.in, khudabukhsh_48@rediffmail.com
[a] Formerly at: Department of Zoology, University of Kalyani, 741235, India.
Received 21 April 2017; revised 11 September 2017; accepted 11 September 2017

caused by Kaposi's sarcoma, or other AIDS-related conditions.[2] Therefore the lung becomes one of the final targets of this virus at later terminal stages of the disease.

Since the advent of AIDS disease in the early 1980s doubt about its viral origin persisted until the actual virus was detected[3] and its viral origin confirmed.[4] But in the first two decades, thousands of people died as no drug could resist the virus from replicating and attacking the immune system through its gene product.

Frantic searches were made initially for drugs that could possibly cure the disease to save the impending global catastrophe, but these proved unsuccessful. Attempts were then made to treat the disease using a defensive and preventive approach, by trying to stop replication of the virus to stall rapid spread of the disease. As reverse transcriptase activity was extremely important for virus replication and entry into the host genome, drugs like reverse transcriptase inhibitors, or protease inhibitors that could interrupt the virus from replicating, showed some response in limiting spread of the virus in the body, but unfortunately their severe side-effects restricted their use. The search was extended to other modes of treatment like complementary and alternative medicines (CAM). In the US, approximately 60% of people with HIV prefer to use various forms of CAM[5] even though the effectiveness of most of these therapies has not yet been firmly established.[6] Some homeopathic remedies have also been claimed to show ameliorative antiviral effects in the treatment and managements of AIDS in humans[7–9] and in animals,[10] but validity of their claims or a molecular mechanism of action in support of their claim still remain unexplored.

Homeopathic nosodes are prepared from microbes, secretions, discharges and tissues, and are quite frequently used in ultra-high dilutions like 30c in a variety of chronic ailments. Use of such nosodes in homeopathy dates back to 1833,[11] but their efficacy has seldom been systematically explored by conducting controlled experimental studies.[12]

Recently one of us (RS) developed an HIV nosode-30c (HIV 30c) from two AIDS-infected volunteers by adopting a widely accepted 15-step protocol.[13] In the current study, an indirect method has been used to test if HIV 30c might show any inhibitory effect on reverse transcriptase activity in cancer cell lines, particularly in lung cancer cells, A549. Allied to this, we made a specific attempt to evaluate regulatory effect, if any, of the HIV nosode on the proliferative activity of growth and division of cells, which are hallmarks of cancer cells, through deployment of various cytotoxicity assay methods including cell viability (MTT assay), cell morphology analysis, Annexin V/FITC assay, DAPI staining, drug−DNA interaction, DNA fragmentation, ROS generation, and MMP determination. Also included were proliferation and senescence assays and expression of pro-apoptotic (Bax, cytochrome c, caspase 3), and anti-apoptotic (Bcl2), telomerase reverse transcriptase (TERT) and topoisomerase II (Top II).

## Materials and methods

### Preparation and source of the nosode

HIV nosode preparation was done by one of us (RS) following the principal guidelines suggested by Samuel Hahnemann,[14] and approved by the Technical Committee of CCRH, New Delhi, Government of India[15,16] and the Homeopathic Guidelines of Drug Proving by the European Committee of Homeopathy through an elaborate 15-step safe method[13] from sera of two confirmed HIV-positive volunteers, by adopting the homeopathic procedure of potentization to obtain the 30c potency (dilution factor $10^{60}$) using water/ethanol as solvent/vehicle after taking the statutory precautionary measures. The volunteers who donated blood samples were confirmed for their HIV types − type I or II. The donors of blood samples also tested negative for Hep B, Hep C, VDRL, Syphilis, etc. Co-infection positive samples, if any, were carefully discarded. Their sera were not included for preparation of the nosode. Viral load HIV-RNA quantification was done by the *in vitro* nucleic acid quantification method (COBAS Taqman) from the central laboratory "Metropolis" in Mumbai. Further, the 30c potencies of HIV nosodes from different batches were tested for viral copies, if any, by RT-PCR, which were found to be negative.[13]

ECH and CCRH guidelines are the only guidelines currently available for homeopathic drug provings, but are generally accepted. Ultradilute potentised homeopathic medicines may be devoid of any active material of the source; and are considered safe for human use, as per the well accepted Homeopathic Pharmacopeia of India (HPI), The Homeopathic Pharmacopeia of the United States (HPUS) (www.hpus.com), and similar other available pharmacopeiae.

### Cell culture

HeLa (cervical cancer), HepG2 (liver cancer), A549 (lung cancer) and normal liver hepatocytes, WRL68 cells were procured from National Centre for Cell Science (NCCS), Pune, India. All cell lines were maintained separately in DMEM containing 10% heat-inactivated FBS and 1% antibiotic mixture for cell culture in a humidified incubator with ambient $O_2$ level and 5% $CO_2$ level at 37°C.

### Cell viability assay

For determination of cytotoxicity against cancer and normal cell lines, HeLa, A549, HepG2 and WRL-68 cells were treated with various concentrations of HIV 30c (0.5 $\mu$l−6 $\mu$l per 100 $\mu$l media) and incubated for 24 h. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (10 $\mu$mol/L) was then added to each well and incubated for 3 h at 37°C. Formazan crystals formed were dissolved in 100 $\mu$l acidic isopropanol and optical density (OD) was measured at 595 nm in an ELISA reader (Thermo scientific, USA).[17] On the basis of MTT assay results, the type of cancer cells showing best response, that is, the maximum number of cancer cells

dying at the minimum dose of HIV nosode, was selected for further detailed experiments.

**Selection of cell line and dose**

A549 cells were selected for further detailed studies, using WRL-68 (normal liver) cells as control. Three different doses were selected, the 50% lethal dose (LD50) (3.5$\mu$l/100 $\mu$l media) and two doses below the LD50 (at 3$\mu$l/100 $\mu$l media and 2.5 $\mu$l/100 $\mu$l media, respectively) for further experiments; Dose-1, -2 and -3 were designated from lowest to highest dose. Control cells were treated with succussed ethanol from the same stock with which the drug was prepared.

**Cell morphological analysis**

Morphology of A549 and WRL-68 cells treated for 24 h with different doses of HIV 30c was studied along with a control group exposed to succussed ethanol alone. Cells were observed under an inverted phase contrast microscope (DMi1 Leica, Germany) equipped with a digital camera.

**Apoptotic analysis**

Treated and control cells were stained with annexin V and PI as described by Matassov *et al.*[18] Fluorescence intensities were determined by fluorescence-activated cell sorting (FACS) using FL-1H filter for annexin V and FL-2H for PI (BD FACSCalibur, USA) to analyze apoptotic cell percentage. Data were analyzed with Cyflogic (v.1.2.1) software.

**Nuclear morphology analysis by DAPI staining**

As additional information, we also studied time-dependent changes in nuclear morphology through fluorescence microscopy.

After 24-h incubation, cells were fixed with 2% paraformaldehyde. Then the cells were stained with DAPI at 10 $\mu$mol/L concentration and observed under a fluorescence microscope (Leica DFC365 FX, Germany).

**Drug–DNA interaction analysis by circular dichroism (CD) spectra**

To ascertain if there was a direct interaction between the drug and ctDNA, circular dichroism data were recorded after keeping the drug at different concentrations from 2 $\mu$l to 5 $\mu$l in contact with naked calf-thymus DNA (ctDNA) and using only untreated ctDNA as reference material. The different CD spectra generated (JASCO J720, Japan) were analyzed using Origin 8 Pro software.

**DNA fragmentation assay**

To further confirm that DNA damage occurred in the A549 cells as a result of HIV-nosode treatment, we also performed the DNA fragmentation assay. For analysis of DNA fragmentation we extracted and further purified genomic DNA using standard protocol.[19] Purified DNA was separated using 1.5% agarose gel electrophoresis and bands were visualized under an ultraviolet trans-illuminator, followed by digital photography.

**Determination of reactive oxygen species (ROS) accumulation**

The increase of ROS in cancer cells is associated with multiple changes in cellular functions such as cell proliferation, migration, differentiation and apoptosis. Cells were collected after drug treatment and fixed with 4% paraformaldehyde. Fixed cells were incubated with 10 $\mu$M $H_2$DCFDA for 20 min in dark. Then the cells were analysed under fluorescence microscope (Leica, Germany) and representative photographs were taken.

**Analysis of changes in mitochondrial membrane potentials (MMP)**

The increase in ROS generation in cancer cells with mitochondrial dysfunction may make cancer cells more vulnerable to further oxidative stress, which in turn pushes cells toward death. After 24 h drug incubation, control and treated cells were fixed with 2% paraformaldehyde and then incubated with 10 $\mu$mol/L rhodamine 123 for 30 min at 37°C in dark. After incubation, cells were immediately analyzed using fluorescence microscopy (Leica, Germany).

**Proliferation assay**

In a cell proliferation assay, we measure the change in the proportion of cancer and normal cells that are dividing. Control and HIV 30c treated cells were harvested after 0–24 h at 3 hourly intervals; cells were washed twice with PBS and trypsinized. The cell suspension was then periodically transferred for cell counting by haemocytometer.

**Migration assay**

To test whether HIV 30c has capability to prevent cancer cells from migrating to invade other tissue (metastasis), we performed migration assay. The confluent A549 cell monolayer was scraped to create a "scratch" with a pipette tip and a first image of the scratches was taken. After treatment with the three different doses of the drug (at dose 1, 2 and 3, respectively) separately, they were subjected to incubation for 24 h and put on the plate and then observed under a phase-contrast microscope; the reference point was duly matched, and photographed regions of the first image were aligned properly and the second image was then acquired.[20]

**Analysis of $\beta$-galactosidase associated senescence**

To detect the cells which are undergoing pre-mature senescence as a consequence of HIV nosode treatment, we conducted the senescence assay.

According to manufacturer's instructions, $\beta$-galactosidase activity was determined by histochemical staining method in HIV 30c treated (3.5$\mu$l/100 $\mu$l media) and control cells at different time intervals.[21]

**Analysis of expressions of proteins related to cytotoxicity by Western blot**

To check apoptosis related protein expressions we seeded A549 cells into 75 mm plates (Tarson, India) at a density of 1 × 105 cells per well. Cells were treated with

different concentrations of HIV 30c separately and incubated for 24 h. An equal amount of protein (50 $\mu$g) was run on 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinyl difluoride membrane. After 3% BSA blocking, the membranes were incubated overnight, at 4°C, with specific primary antibodies, including p53, telomerase reverse transcriptase (TERT), which is necessary for telomere synthesis (required for repeated cancer cell division as well as retro-virus replication), Bcl-2, Bax, caspase-3, cytochrome c or GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The membrane was then incubated for 2 h with alkaline phosphatase (ALKP)-conjugated secondary antibody. 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium was used as developer and protein concentration was quantified by densitometry using image J software.

### Analysis of Type II Topoisomerases (Top II) activity by confocal microscopy

Expression of Top II protein is an important marker associated with DNA replication and most anti-cancer drugs are known to reduce the expression of Top II that helps in retaining the DNA damage and thereby pushing the cells towards DNA-damage linked apoptosis.

HIV 30c treated cells at three doses were separately incubated for 24 h with primary antibody (Santa Cruz Biotechnology, USA) at 4°C overnight and developed with secondary FITC conjugated anti-Top II antibody. Then the cells were stained with DAPI at 10 $\mu$mol/L concentration and photographs were taken under a confocal microscope (Carl Zeiss LSM 510 META Laser Scanning Microscope).

### Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post-hoc tests, using SPSS 20 software (Statistical Package for the Social Sciences Inc., IBM, USA) to identify if the differences were significant among the mean-values of different groups. All experiments were done in triplicates and replicated thrice. Results were expressed as mean ± standard error of mean (SEM). $P < 0.05$ was considered as significant.

## Results

### Effects of HIV 30c on the survival of A549 and WRL-68 cells

Cell viability was gradually decreased in a dose dependent manner. HIV 30c reduced viability of Hela, HepG2 and A549 cells with different doses. In 24 h incubations, 50% cell death occurred at the dose of 4.47, 4.71 and 3.5$\mu$l/100 $\mu$l media on Hela, HepG2 and A549 cell lines, respectively. However, cytotoxicity was found to be significantly greater in A549 cells. Experimental results for the normal cell line (WRL-68) revealed that the cytotoxicity produced by HIV 30c after 24 h of treatment was relatively much less (Figure 1 and Supplementary Figure 1). In all cases, results of the succussed alcohol "placebo" treated cells did not show any appreciable modulating effects.

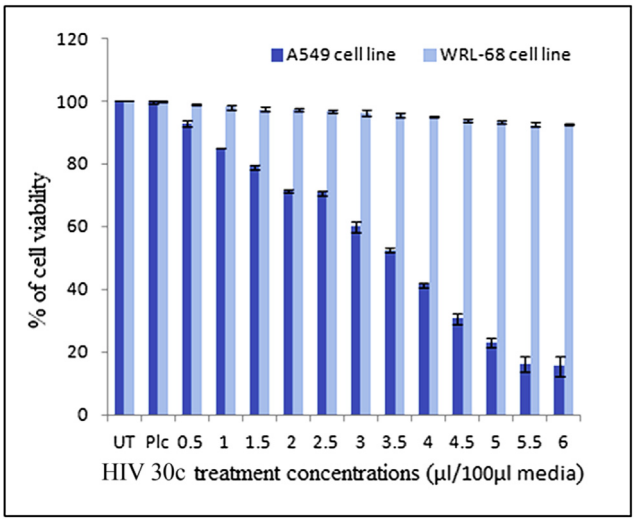



**Figure 1 Cell viability assay:** Cells (A549 and WRL-68) were treated with 0.5 $\mu$l–6 $\mu$l of HIV 30c per 100 $\mu$l media for 24 h. Cell viability was determined by MTT assay. The results showed gradual reduction in the viability of A549 celline. HIV 30c produced negligible cytotoxicity in normal cell line (WRL-68) after 24 h of treatment.

### Morphological changes in A549 and WRL-68 cells

Morphological changes were observed in A549 cells treated with HIV 30c in the form of rounding off of the cytoplasmic periphery along with gradual detachment of cells from the substrate. Shrinkage of cell and blebbing of cell membrane were found in treated cells (Figure 2A). WRL-68 cells showed normal cell architecture as compared to controls after HIV 30c treatment (Figure 2B).

### Apoptosis analysis by FACS

Cells showed distinct positive binding with annexin V when treated with HIV 30c, indicating movement of phosphatidyl serine to the outer cell surface. Treatment with HIV 30c in A549 cells caused significant apoptotic cell death as compared to control set (Figure 3A). Dot plot suggested that more or less the same number of living cells were present in the HIV 30c treated WRL-68 cells after 24 h incubation as compared to the untreated control (Figure 3B).

### Nuclear changes

After DAPI staining, nuclear condensation was observed in A549 cells treated with the different doses of the drug (Supplementary Figure 2A) in a time-dependent manner while the WRL-68 cells did not show such notable changes in nuclear condensation (Supplementary Figure 2B). This result suggested that HIV 30c could potentially initiate the process of cellular DNA damage by commencing with the nuclear condensation process. DNA damage was initiated at 8th h with the drug treatment.

### Changes in circular dichroism (CD) spectra

Peak shifts in CD spectra indicated that changes took place in ct-DNA helical structure in the drug treated series in A-549 cells as a result of drug–DNA interaction (Figure 4A).

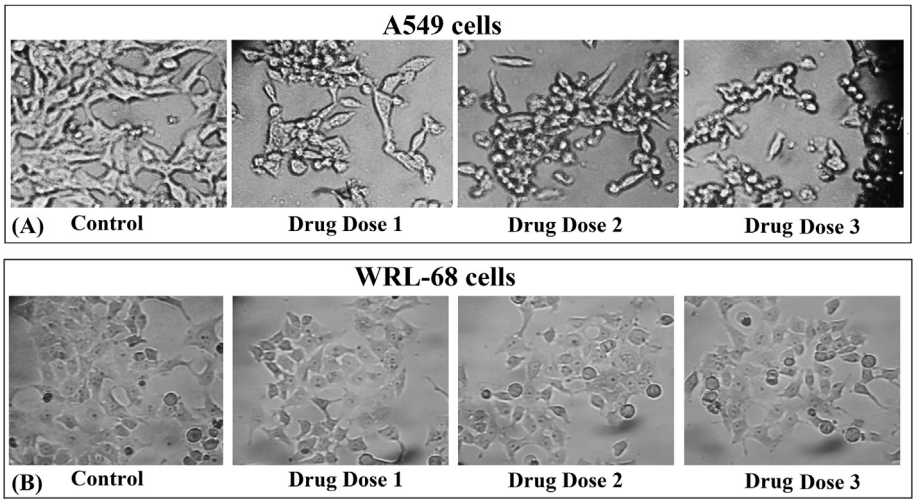
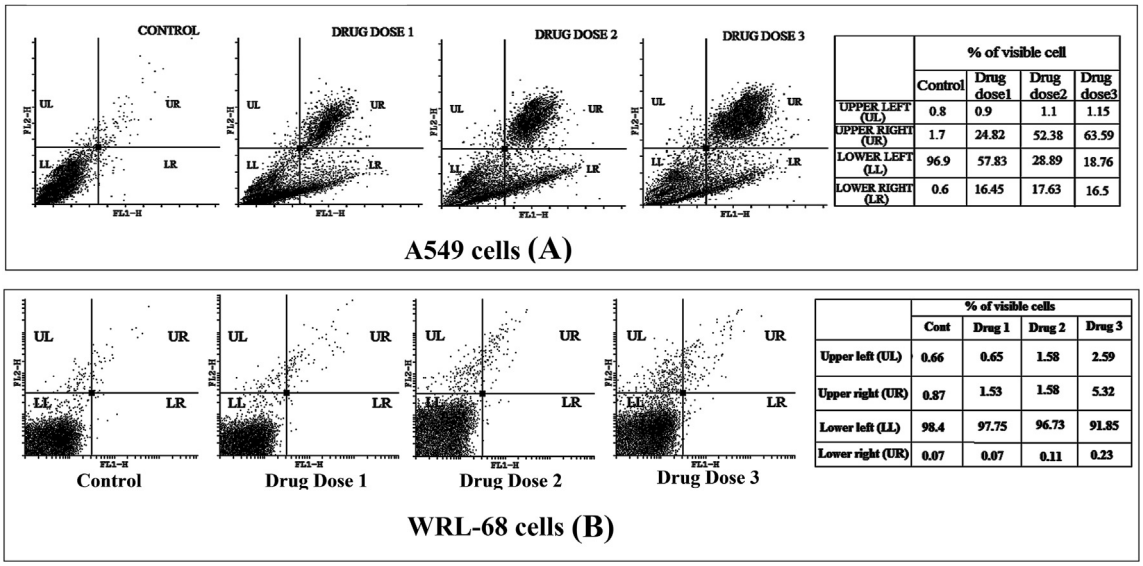



**Figure 2 Morphological analysis of (A) A549 and (B) WRL-68 cells:** A549 cells showed membrane blebbing, cell periphery shrinkage and rounding off of cells in the HIV 30c treatment group. WRL-68 cells showed normal cell architecture as compared to control set after HIV 30c treatment.




| | % of visible cell | | | |
|---|---|---|---|---|
| | Control | Drug dose1 | Drug dose2 | Drug dose3 |
| UPPER LEFT (UL) | 0.8 | 0.9 | 1.1 | 1.15 |
| UPPER RIGHT (UR) | 1.7 | 24.82 | 52.38 | 63.59 |
| LOWER LEFT (LL) | 96.9 | 57.83 | 28.89 | 18.76 |
| LOWER RIGHT (LR) | 0.6 | 16.45 | 17.63 | 16.5 |

| | % of visible cells | | | |
|---|---|---|---|---|
| | Cont | Drug 1 | Drug 2 | Drug 3 |
| Upper left (UL) | 0.66 | 0.65 | 1.58 | 2.59 |
| Upper right (UR) | 0.87 | 1.53 | 1.58 | 5.32 |
| Lower left (LL) | 98.4 | 97.75 | 96.73 | 91.85 |
| Lower right (UR) | 0.07 | 0.07 | 0.11 | 0.23 |

**Figure 3 (A) Annexin V/PI assay in A549 cells:** A549 Cells were undergoing apoptosis after HIV 30c treatment. Dot plot suggested that more number of apoptosis occurred at the highest dose of HIV 30c after 24 h incubation [Upper left = dead cells, lower left = live cells, upper right = late apoptotic cells and lower right = early apoptotic cells]. **(B) Annexin V/PI assay in WRL-68 cells:** Dot plot suggested that more or less the same number of living WRL-68 cells were present in HIV 30c treatment set after 24 h incubation [Upper left = dead cells, lower left = live cells, upper right = late apoptotic cells and lower right = early apoptotic cells].

### HIV 30c induced DNA fragmentation in A549 cells

HIV 30c treatment induced cellular DNA fragmentation leading to DNA damage as observed from different fragmented bands of DNA incubated with HIV 30c and compared to that of untreated cells. Experimental analysis also suggested initiation of cell DNA fragmentation in HIV 30c treated A-549 cells (Supplementary Figure 3). Since there were no nuclear condensation and changes in Top II expression noted in the WRL-cells on HIV-nosode treatment, the DNA fragmentation study in this cell line was considered unnecessary and therefore not done.

### Changes of intracellular ROS generation

Microscopic photographs (Figure 4B) would depict an increase in intensity of bluish-green colour in the drug treated sample; this would implicate the induction of greater quantity of ROS in the *HIV 30c* treated cells. In the case of the treatment group, ROS generation increased in a dose-dependent manner. But in WRL-68 cell line after *HIV 30c* treatment no significant change in ROS generation was found (Figure 4C).

### Mitochondrial membrane potential analysis results

There was a decrease in mitochondrial membrane potential (MMP) in a dose-dependent manner after treatment, reaching the maximum at dose 3.5$\mu$l/100 $\mu$l media of *HIV 30c* treatment in A549 cells (Figure 5A) but not in WRL-68 cells (Figure 5B).

### Changes in cell proliferation rate

Analysis of the cell proliferation histogram revealed that HIV 30c treatment reduced A-549 cell growth as compared

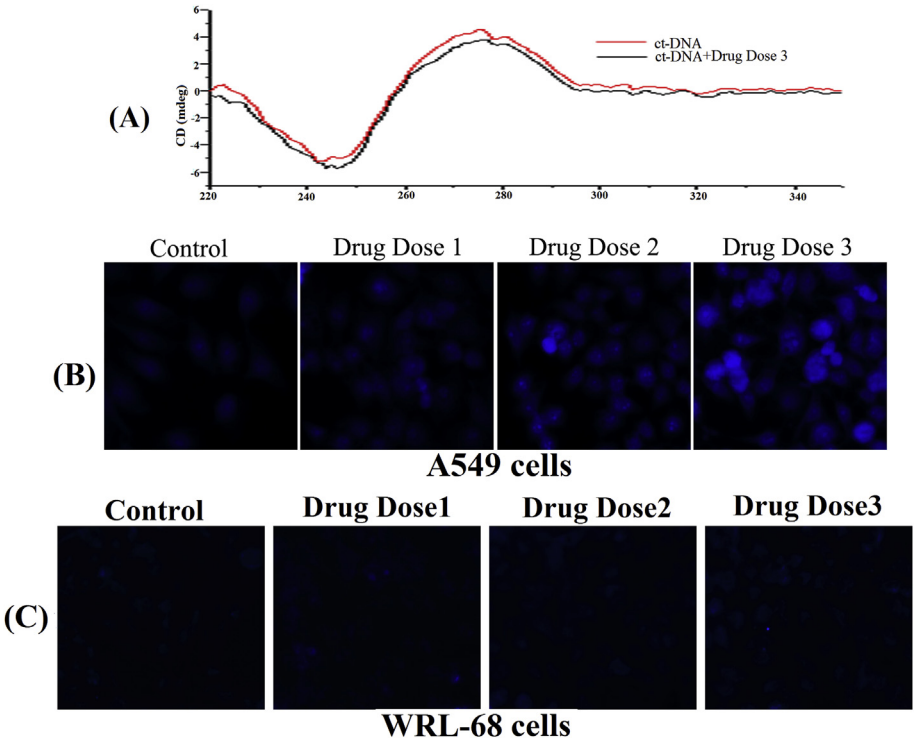



**Figure 4 (A) Drug–DNA interaction:** Circular dichroism spectra indicated HIV 30c can interact with ct-DNA. **(B) and (C) Fluorescence images of ROS in A549 cells and WRL cells after HIV 30c treatment:** Accumulation of ROS was increased in a dose-dependent manner after HIV 30c treatment in A549 cells. No change in reactive oxygen species (ROS) accumulation was found at even highest dose of HIV 30c treatment for WRL-68 cells. See Supplementary file 2 for relevant statistical analysis and bar-graphs.

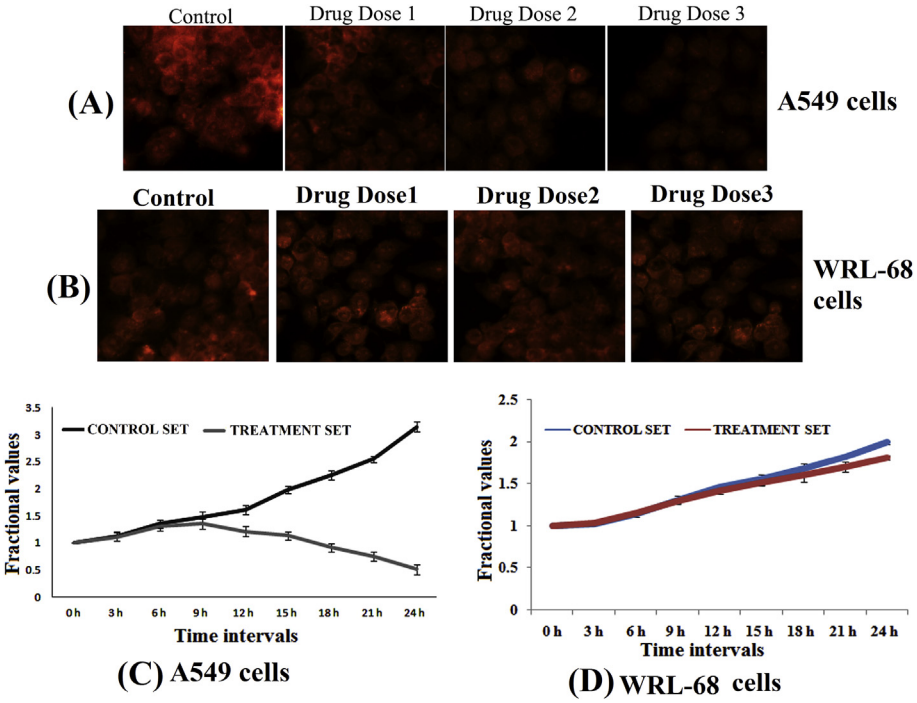



**Figure 5 (A) Depolarization of mitochondrial membrane potential (MMP**): fluorescence intensity was reduced gradually at increasing HIV 30c doses, indicating gradual reduction of MMP in A549 cells. **(B)** No change was observed in depolarization of mitochondrial membrane potential (MMP) after WRL-68 cells were treated with HIV-nosode. See Supplementary file 2 for relevant statistical analysis and bar-graphs. **(C)** This indicated the reduction in proliferative property of A549 cells upon treatment with HIV 30c at the dose 3.5$\mu$l/100 $\mu$l media compared to that of the control plates at different time points. Lowest proliferation was reached at 24 h of treatment. **(D)** Data of proliferation assay indicated no significant amount of reduction in cell division of WRL-68 cells upon treatment with HIV 30c at dose 3.5$\mu$l/100 $\mu$l in the media compared to that of the control plates at different time points.

to control sets. In early hours of treatment with HIV 30c, A549 cellular proliferation was evident but after 9th h of treatment, it gradually decreased until the 24th h of HIV 30c treatment (Figure 5C) when it touched the lowest level. No significant reduction in cell division of WRL-68 cells was noted in plates treated with HIV 30c at a dose of 3.5$\mu$l/100 $\mu$l in media as compared to that of the untreated control cells at different time points (Figure 5D).

**Efficacy of HIV 30c in prevention of cell migration**

Cell migration gradually reduced upon treatment with the increase in drug dose. In case of positive control the migratory index reduced to 65.84 $\pm$ 1.9 from the reference plate (100.13 $\pm$ 1.3). In the case of the different drug-treated cell series (2.5$\mu$l/100 $\mu$l, 3$\mu$l/100 $\mu$l and 3.5$\mu$l/100 $\mu$l media of HIV 30c), the migratory indices gradually increased to 107.12 $\pm$ 1.89, 112.40 $\pm$ 2.25 and 142.32 $\pm$ 0.79, respectively, indicating thereby a corresponding decrease in cell migration (Figure 6A). There was no visible change in cell migration in the WRL-68 cells upon HIV-nosode treatment, as expected since they were derived from normal cell lines.

**Results of senescence assay in A549 cells and WRL-cells treated with HIV 30c**

HIV 30c induced premature senescence in lung cancer cells (A549) in a time-dependent manner. HIV 30c treated A549 cells showed positive $\beta$-galactosidase activity. Blue colour increased in a time dependent manner with the maximum at 20th h (Figure 6B). These features were not observed in the WRL-68 cell line on HIV-nosode treatment (Figure 6C).

**Change in expressions of different proteins related to cytotoxicity**

After HIV 30c treatment, expression of proteins related to cell survival and apoptosis was quantified in A549 cells. Expressions of p53 and Bax were up-regulated in HIV 30c treated cells. On the other hand, the expressions of TERT and Bcl2 decreased upon treatment of HIV 30c, showing clearly its inhibitory effect on reverse transcriptase activity required for both telomere synthesis and retro-virus replication. Further cytochrome c and caspase 3 expressions were increased in HIV 30c treated A549 cells in a dose dependent manner (Figure 7).

**Changes in Top II expression**

Decreased levels of Top II in the cellular nucleus were shown by confocal microscopy. Experimental results indicated that Top II expression was reduced in the nucleus of the drug-treated group (2.5$\mu$l/100 $\mu$l, 3$\mu$l/100 $\mu$l and 3.5$\mu$l/100 $\mu$l media of HIV 30c), when compared with control set (Figure 8A). There was no corresponding change in expression of Top II in the WRL-cells on HIV-nosode treatment (Figure 8B).

## Discussion

While HIV 30c treatment at LD50 dose (that is, 3.5$\mu$l/100 $\mu$l media) reduced the viability of A549 cells to 50%, it reduced the viability of WRL-68 by 4% only. This fact clearly differentiates the action of the nosode from that of many other conventional chemotherapeutic drugs, which show considerable cytotoxic effects in both cancer and normal cells alike. HIV 30c successfully

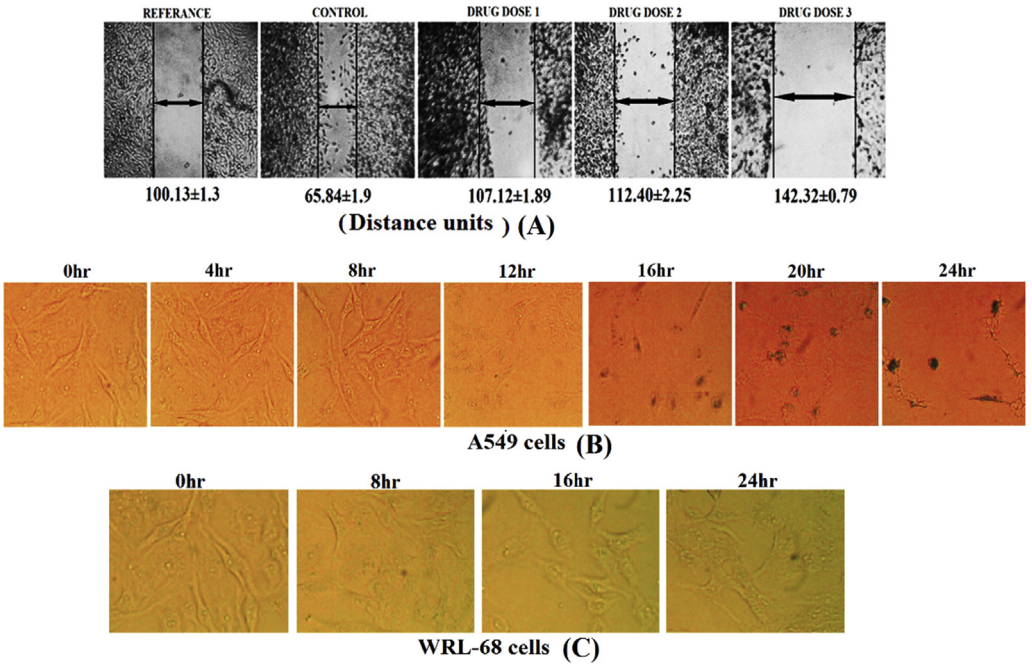



**Figure 6 (A) Migration assay:** The clear zone in the control plate was reduced compared to the corresponding zone of the reference plate after the 24 h treatment. But in the case of the drug doses, the clear zone was increased compared to that seen in the reference plate. The graph represents the migratory distance of the cells with respect to the reference plate. The results are represented as mean $\pm$ SE (significance *P < 0.05). **(B) Senescence assay:** The induction of premature senescence was initiated at 12th h after drug treatment in A549 cells. **(C)** Induction of premature senescence was not observed up to 24 h after treatment of WRL-68 cells with HIV-nosode treatment.

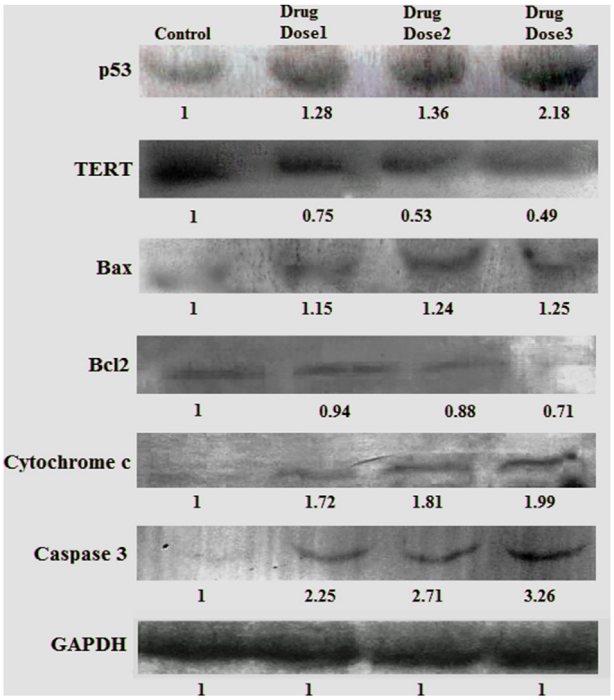



**Figure 7 Analytical data and images of Western blots**: p53, Bax, cytochrome c, and caspase 3 activities were up-regulated and TERT and Bcl-2 expressions were down-regulated by HIV 30c treatment after 24 h incubation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as loading control.

induced an apoptotic response in A549 cells, as evident from the external morphological changes of cells, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation, with the viability of the cancer cells being reduced in a dose-dependent manner.

When cells are transformed into cancer, they become endowed with the property of auto-replication and immortality. They also gradually become unresponsive to any action affecting their divisional activities by interfering with or inhibiting cell division. They continue to divide and re-divide and then start moving to other tissue (metastasis) to invade. Thus the primary target of many anticancer drugs is the DNA, the main machinery that is involved in cell division. The strategies of most anticancer action are two-fold: either they prevent the uncontrolled cell division or they kill those unruly cells that cannot be prevented from dividing through apoptosis (or autophagy or other means of cell killing like necrosis). Thus research directed towards different events of cell division and progression through apoptosis can give important clues about the potential of any drug meant for combating cancer. We focus on the action of HIV nosode to pinpoint how far it succeeded in stopping proliferation activity and how far it might contribute to channel the erring cancer cells towards their death, some of the landmarks for this being abnormal cell morphology, nuclear chromatin condensation, ROS generation, ct-DNA binding ability, senescence status, and other apoptosis markers.

Loss of plasma membrane asymmetry is an early feature of apoptosis progression. In apoptotic cells, the membrane phospholipid phosphatidyl serine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is Ca2+-dependent phospholipid-binding protein with high affinity for PS, and binds to the exposed apoptotic cell surface PS.[22] Results of annexin V/FITC assay confirmed that HIV 30c treatment induced apoptosis with externalization of phosphatidyl serine.

Through metastasis, cancer cells invade other tissue/organs, creating a major problem in surgical/radiological treatment of cancer and threatening survival of patients.[23]

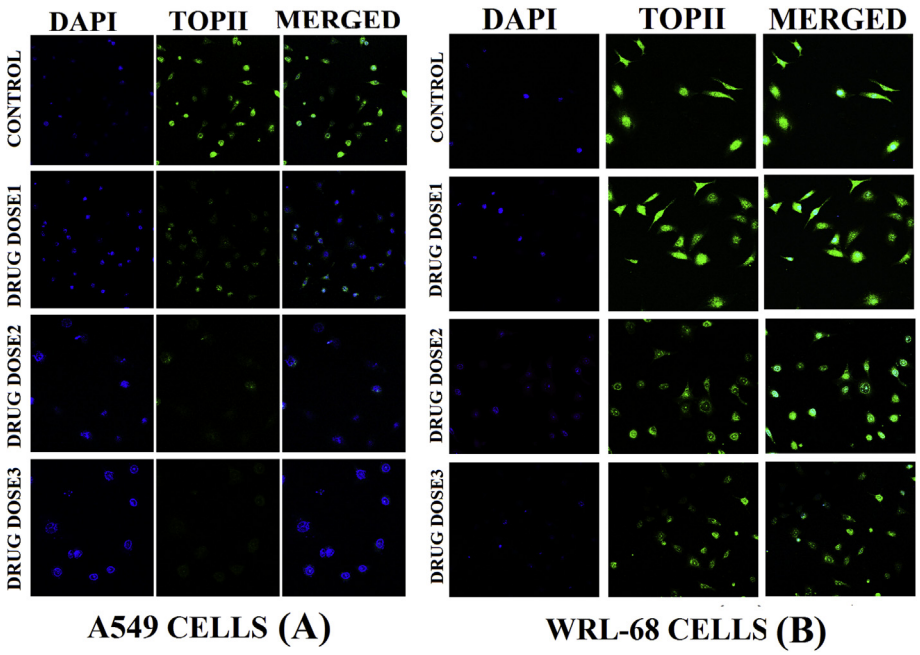



**Figure 8 (A) Confocal microscopic analysis:** Expression of Top II in the nuclei of A549 cells was studied after treatment with HIV 30c. Changes of expression of Top II were observed in the drug treated group as compared to control. **(B)** No change of Top II expression was noted in the nuclei of WRL-68 cells after HIV 30c treatment.

Results of migration assay can reflect on metastatic status of cancer cells to a considerable extent. Results of migration assay of A549 cells revealed that HIV 30c treatment reduced the migratory property of A549 cells.

Type II topoisomerases (Top II) constitute a family of nuclear enzymes essential for all living cells. These enzymes are capable of transferring one double helix through a transient break in another DNA double helix. Top II play important roles in DNA metabolic processes, in which they are involved in DNA replication, transcription, chromosome condensation and de-condensation.[24,25] Top II are also the cellular target for a number of widely used anticancer agents currently in clinical use.[26] Down-regulation of Top II expression in A549 cancer cells after HIV 30c treatment indicated that this nosode has ability to act as a topoisomerase inhibitor, contributing to DNA damage, and interfering with divisional activities, thus pushing the cells further towards death.

As stated, DNA is the molecular target for many drugs that are used in cancer therapeutics. Results of nuclear condensation of cancer cells after administration of the drug made us further interested to investigate if the drug could potentially bind with DNA. CD data confirmed that the ultra-highly diluted drug still had an ability to interact with DNA and change the ellipticity of the DNA-helices, bringing about conformational changes. DNA fragmentation data also supported ability of the drug to induce cellular DNA fragmentation, which is considered a hallmark of apoptosis. Incidentally, DNA fragmentation assay indicated a positive relationship between drug dose and rate of DNA damage in cancer cells. Accurate repair of DNA double-strand breaks is essential to life. However, any inhibition of Top II by a drug could interfere with DNA double-strand breaks, leading to further toxicity and could cause large-scale sequence rearrangements that in turn could act as a factor for promoting premature ageing and cancer.[27,28]

This interpretation also finds support from ROS generation data. Interaction of ROS with DNA can produce DNA damage, which induces the cells to move towards premature senescence with active involvement of elevation of p53 expression, resulting in an increase in $\beta$-galactosidase activity. HIV 30c induced generation of ROS that can further damage DNA integrity, which in turn could lead cells to a state of premature senescence. Senescence limits the life span and proliferative capacity of cancer cells; therefore induction of senescence is regarded as an important mechanism of cancer prevention and spread. DNA damage was initiated at 8th h upon drug treatment and a senescence state was initiated at around 12th h. These findings could be taken as a clue to suggest that DNA damage might actually contribute to the initiation process of premature senescence in the A549 cells upon HIV 30c treatment.

Further, cell senescence is broadly defined as the physiological program of terminal growth arrest, which can be triggered by alterations of telomeres.[29] Telomerase lengthens telomeres in DNA strands and commonly expressed in human cancer cells. Increased telomerase expression produces vulnerability of cancer cells, distinguishing them from normal cells in the body. Recent studies also suggest that it is implicated in tumour progression in other unexpected ways.[30,31] These observations led us to investigate if the drug could inhibit telomerase synthesis in cancer cells. In this study, HIV 30c was found to down-regulate expression of telomerase reverse transcriptase (TERT) which is the catalytic sub-unit of telomerase. Therefore, we emphasize the results of TERT expression which we believe to be of great significance; it implies that the inhibition of TERT expression by HIV-nosode is serving a dual role: against both cancer and retro-virus growth. The results would indicate that HIV-nosode could inhibit division of cancer cells and retrovirus alike, and it could also play a role in inducing apoptosis in cancer cells.[32]

MMP is considered as another such phenomenon that is directly related to drug-induced modulation of ROS-production. Treatment with HIV 30c resulted in a considerable increase in ROS generation and corresponding decrease in MMP, supporting earlier research data on anti-cancer drugs.[33]

To further test whether the apoptotic process was activated via mitochondrial intrinsic pathway, we also investigated the status of cytochrome c release, associated with changes in Bax/Bcl2 protein ratio in drug-treated cancer cells. Alteration in the level of Bax and Bcl-2 helped us to determine whether cells would undergo apoptosis leading to cell death, or be directed towards the survival pathway.[33] Down-regulation of Bcl-2 and up-regulation of Bax protein in the drug-treated cells would suggest that the possible molecular mechanism through which HIV 30c induced apoptosis in A549 cells could involve this pathway. This probably resulted in the release of cytochrome c and other pro-apoptotic factors from the mitochondria, leading to activation of caspase-cascade. The signalling cascade clearly indicated that HIV 30c nosode has potential to turn on the mitochondrial apoptotic intrinsic pathway in A549 cells.

In this study we followed action of the nosode in *in vitro* culture conditions: that is, the effect of the drug must have been directly on the free cells without intervention of other tissues or hormones for example. Since the nosode HIV 30c was diluted by a factor of $10^{60}$, the possibility of presence of even a single original drug molecule is extremely low, yet it showed its apparent ability to modulate the expression of well-known cancer bio-markers in a given time span, particularly noticeable in the cancer cells, though less so in the normal WRL-68 cells, while the succussed alcohol ("placebo") without having any drug molecular imprints failed to do so in the initial control parameters tested. It seems that homeopathic potentized remedies may have the ability to trigger the "target"/"master gene(s)" and modulate precisely the action of some specific and relevant genes downstream in a "chain", reaction, pushing them into either activation or inactivation, in a regulatory manner to restore their proper functional activities, as advocated by Khuda-Bukhsh[34–40] and also supported recently by Paolo Bellavite and his colleagues.[41,42] However, precise details of the mechanism by which this

initial trigger is implemented needs further experimental elucidation.

Conventional cancer treatments mostly utilize three principal weapons to combat the disease: by directly ablating them with radiation therapy; poisoning them with chemotherapy; or removing them altogether through surgery. While these methods of treatment become indispensable depending on the state of cancer, each method has its own limitation, because along with cancer cells, a large percentage of normal cells are also adversely affected, often causing severe side-effects. Besides, sometimes the methods of chemotherapy and radiotherapy also cause great harms to the immune system which make it vulnerable to other diseases. This probably explains why there is a surge in accepting CAM as a desired alternative, even though the effectiveness of most of these therapies has not yet been scientifically established.[37] Research authentication would give more confidence to both doctors and patients, when CAM is being used at least as a supportive therapy, because it has no or little toxic side-effects of its own and still can give a better way of life to terminally ill patients.

## Conclusion

From the results of the present *in vitro* controlled pilot study on HIV 30c nosode, the following inferences may be drawn: i) HIV nosode administration could cause cytotoxicity in several cancer cell lines, of which it had stronger cytotoxic effects against A549 cells; ii) it showed ability of potential intervention at all levels of activities towards combating division and progression of cancer; iii) most importantly, it triggered the requisite molecular mechanism to inhibit telomerase reverse transcriptase and Top II activities, and induced cell death by triggering the pro-apoptotic genes, thereby restricting their divisional dynamics.

## Acknowledgements

Part of the work was financed through a Senior Research Fellowship (F1-17.1/2012-13/MANF-2012-13-MUS-WES-13662/(SA-III/Website) Dated 28-Feb-2013) grant of UGC, Government of India, sanctioned to JM, and part through Emeritus Fellowship (No. F.6-6/2013-14/EMERITUS-2013-14-GEN-2067/(SA-II) dt. 18th April, 2014) from a grant provided to ARK-B, Zoology, Kalyani University, for his kind help in performing the confocal microscopy.

### Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.homp.2017.09.001.

## References

1 Sepkowitz KA. AIDS—the first 20 years. *N Engl J Med* 2004; **344**: 1764–1772, http://www.nejm.org/doi/full/10.1056/NEJM200106073442306#t=article.
2 Kirch W. *Encyclopedia of public health*. New York: Springer 2008, pp 676–677, https://books.google.co.in/books?id=eSPK7-CHw7oC&pg=PA676&redir_esc=y#v=onepage&q&f=false.
3 Barré-Sinoussi F, Chermann JC, Rey F, *et al*. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983;868–871 [Pubmed], https://www.ncbi.nlm.nih.gov/pubmed/6189183.
4 Conte JE, Hadley WK, Sande M. Infection-control guidelines for patients with the acquired immunodeficiency syndrome (AIDS). *N Engl J Med* 1983; **309**: 740–744.
5 Littlewood RA, Vanable PA. Complementary and alternative medicine use among HIV-positive people: research synthesis and implications for HIV care. *AIDS Care* 2008; **20**: 1002–1018 [Pubmed], https://www.ncbi.nlm.nih.gov/pubmed/18608078.
6 Lutge EE, Gray A, Siegfried N. The medical use of cannabis for reducing morbidity and mortality in patients with HIV/AIDS. *Cochrane Database Syst Rev* 2013;30. CD005175. [Pubmed], https://www.ncbi.nlm.nih.gov/pubmed/23633327.
7 Ullman D. *Discovering homeopathy: medicines for 21st century*. Revised edn. Berkeley, California 94712: North Atlantic books, 1991, p. 258.
8 Rastogi DP. Asymptomatic HIV carriers. *Br Homoeopath J* 1994; **83**: 54.
9 Rastogi DP, Singh VP, Singh V, *et al*. Homeopathy in HIV infection: a trial report of double-blind placebo controlled study. *Br Homoeopath J* 1999; **88**: 49–57 [Pubmed], https://www.ncbi.nlm.nih.gov/pubmed/10335412.
10 Singh LM, Gupta G. Antiviral efficacy of homoeopathic drugs against animal viruses. *Br Homoeopath J* 1985; **74**: 168–174.
11 Allen HC. *Materia Medica of nosodes*. New Delhi: B. Jain Publ., 1988, p. 139.
12 Mondal J, Samadder A, Khuda-Bukhsh AR. Psorinum 6× triggers apoptosis signals in human lung cancer cells. *J Integr Med* 2016; **14**: 143–153, http://www.jcimjournal.com/jim/showReferPage.aspx?articleTitle=Psorinum+6%C3%97+triggers+apoptosis+signals+in+human+lung+cancer+cells.
13 Shah RHIV. nosode:The human pathagenetic trial. *Forsch Komplementmed* 2015; **22**: 156–162 [Pubmed], https://www.ncbi.nlm.nih.gov/pubmed/26335635?dopt=Abstrac.
14 Hahnemann S. *Organon of medicine*. 6th edn. New Delhi: B. Jain Publ, 1979, pp 190–212.
15 Central Council for Research in Homeopathy: general directions and instructions for proving of drugs. http://ccrhindia.org/drugprovingintro.asp. Accessed 11 June 2015.
16 European Committee for Homeopathy: homeopathic drug proving guidelines. www.homeopathyeurope.org/publications/guidelines/homeopathicprovings/ECH_Proving_Guidelines-vl.pdf. Accessed 11 June 2015.
17 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
18 Matassov D, Kagan T, Leblanc J, *et al*. Measurement of apoptosis by DNA fragmentation. *Methods Mol Biol* 2004; **282**: 1–17.
19 Bhattacharyya SS, Paul S, De A, *et al*. Poly (lactide-co-glycolide) acid nanoencapsulation of synthetic coumarin: cytotoxicity and bio-distribution in mice, in cancer cell line and interaction with calf thymus DNA as target. *Toxicol Appl Pharmacol* 2011; **253**: 270–281.
20 Hulkower KI, Herber RL. Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics* 2011; **3**: 107–124.
21 Itahana K, Campisi J, Dimri GP. Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. *Methods Mol Biol* 2007; **371**: 21–31.
22 van Engeland M, Ramaekers FC, Schutte B, *et al*. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry* 1996; **24**: 131–139.
23 Klein CA. Cancer. The metastasis cascade. *Science* 2008; **321**: 1785–1787.

24 Giaccone G. DNA topoisomerases and topoisomerase inhibitors. *Pathol Biol Paris* 1994; **42**: 346−352.
25 Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 2002; **3**: 430−440.
26 Willmore E, de Caux S, Sunter NJ, *et al*. A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia. *Blood* 2004; **103**: 4659−4665.
27 Jackson SP. Sensing and repairing DNA double-strand breaks. *Carcinogenesis* 2002; **23**: 687−696.
28 Abbotts R, Thompson N, Madhusudan S. DNA repair in cancer: emerging targets for personalized therapy. *Cancer Manag Res* 2014; **6**: 77−92.
29 Roninson IB. Tumor cell senescence in cancer treatment. *Cancer Res* 2003; **63**: 2705−2715.
30 Kim NW, Piatyszek MA, Prowse KR, *et al*. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; **266**: 2011−2015.
31 Kyo S, Inoue M. Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy? *Oncogene* 2002; **21**: 688−697.
32 Shay JW, Zou Y, Hiyama E, *et al*. Telomerase and cancer. *Hum Mol Genet* 2001; **10**: 677−685.
33 Liu Y, Jiao R, Ma Z, *et al*. Sanguinarine inhibits angiotensin II-induced apoptosis in H9c2 cardiac cells via restoring reactive oxygen species-mediated decreases in the mitochondrial membrane potential. *Mol Med Rep* 2015; **12**: 3400−3408.
34 Khuda-Bukhsh AR. Potentized homeopathic drugs act through regulation of gene-expression: a hypothesis to explain their mechanism and pathways of action in vivo. *Complement Ther Med* 1997; **5**: 43−46.
35 Khuda-Bukhsh AR. Towards understanding molecular mechanisms of action of homeopathic drugs: an overview. *Mol Cell Biochem* 2003; **253**: 339−345.
36 Mallick P, Mallick JC, Guha B, Khuda-Bukhsh AR. Ameliorating effect of microdoses of a potentized homeopathic drug, Arsenicum Album, on arsenic-induced toxicity in mice. *BMC Complement Altern Med* 2003; **3**: 7. Oct 22.
37 Khuda-Bukhsh AR. Laboratory research in homeopathy. *Pro Integr Cancer Ther* 2006; **5**: 320−332.
38 Khuda-Bukhsh AR. Mice as a model for homeopathy research. *Homeopathy* 2009; **98**: 267−279.
39 Khuda-Bukhsh AR. Current trends in ultra-high dilution research with particular reference to gene regulatory hypothesis: review. *The Nucleus* 2014; **57**(1): 3−17 https://doi.org/10.1007/s13237-014-0105-0.
40 Khuda-Bukhsh AR. An overview of research at University of Kalyani in exploring some basic issues of Homoeopathy. *Indian J Res Homoeopathy* 2017; **11**: 147−157.
41 Bellavite P, Signorini A, Marzotto M, *et al*. Cell sensitivity, non-linearity and inverse effects. *Homeopathy* 2015; **104**: 139−160.
42 Marzotto M, Bonafini C, Olioso D, *et al*. Arnica montana stimulates extracellular matrix gene expression in a macrophage cell line differentiated to wound-healing phenotype. *PLoS One* 2016; **11**: e0166340.