Science Press

Journal homepage:
www.jcimjournal.com/jim
www.elsevier.com/locate/issn/20954964
Available also online at www.sciencedirect.com.

• Research Article

A homeopathic nosode, Hepatitis C 30 demonstrates anticancer effect against liver cancer cells *in vitro* by modulating telomerase and topoisomerase II activities as also by promoting apoptosis via intrinsic mitochondrial pathway

Jesmin Mondal[1], Jayeeta Das[1], Rajesh Shah[2], Anisur Rahman Khuda-Bukhsh[1]

1. Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani-741235, India
2. Life Force, 411 Krushal Commercial Complex, Chembur, Mumbai-400089, India

ABSTRACT

OBJECTIVE: Homeopathic nosodes have seldom been scientifically validated for their anticancer effects. This study was conducted to examine if a recently developed hepatitis C nosode has demonstrable anticancer potential in cancer cells *in vitro*.

METHODS: Anticancer effects of Hepatitis C 30C (Hep C 30), if any, were initially tested on three cancer cell lines, HepG2 (liver cancer), MCF-7 (breast cancer) and A549 (lung cancer) and one normal liver cell line WRL-68 cells and subsequently a more thorough study using further scientific protocols was undertaken on HepG2 cells (against WRL-68 cells as the normal control) as HepG2 cells showed better anticancer response than the other two. Three doses, one at 50% lethal dose (LD_{50}) and the other two below LD_{50}, were used on HepG2 cells subsequently. Protocols like apoptosis induction and its possible signaling mechanism were deployed using immunoblots of relevant signal proteins and confocal microscopy, with particular reference to telomerase and topoisomerase II (Top II) activities, two strong cancer biomarkers for their direct relationship with divisional activities of cells and DNAs.

RESULTS: Hep C 30 induced apoptosis, caused distorted cell morphology typical of apoptotic cells, increased reactive oxygen species generation and produced increased DNA nicks. Further it enhanced pro-apototic signal proteins like Bax, cytochrome *c* and inhibited anti-apoptotic signal proteins, Bcl-2, cytochrome *c* and caspase-3, changed mitochondrial membrane potential and caused externalization of phosphatidylserine. The drug also decreased expression of two cancer biomarkers, Top II and telomerase, consistent with its anticancer effect.

CONCLUSION: Hep C 30 has demonstrable anticancer effects against liver cancer cells *in vitro*.

Keywords: formularies, homeopathic; antineoplastic agents; telomerase; DNA topoisomerases, type II; membrane potential, mitochondrial

http://dx.doi.org/10.1016/S2095-4964(16)60251-0
Received December 23, 2015; **accepted** March 22, 2016.
Correspondence: Prof. Anisur Rahman Khuda-Bukhsh; E-mail: prof_arkb@yahoo.co.in, khudabukhsh_48@rediffmail.com

Citation: Mondal J, Das J, Shah R, Khuda-Bukhsh AR. A homeopathic nosode, Hepatitis C 30 demonstrates anticancer effect against liver cancer cells (HepG2) *in vitro* by modulating telomerase and topoisomerase II activities as also by promoting apoptosis via intrinsic mitochondrial pathway. *J Integr Med*. 2016; 14(3): 209–218.

1 Introduction

Nosodes are highly diluted homoeopathic preparations sourced from biological materials such as diseased tissues, organisms, cultures (bacteria, fungi and viruses), or parasites, or from decomposed products from humans or animals. In homeopathic practices, more than 45 major nosodes have been in use since 1830[1,2]. Though clinical benefits of nosodes have often been experienced by homeopathic practitioners[3,4] and patients, very few have so far been scientifically tested for their anticipated effects, particularly *in vitro*[5]. The 30th potency (30C) of the hepatitis C nosode (Hep C 30), prepared by following homeopathic principle of succussion and dilution, is one such nosode successfully developed recently from genotypes I and III of the hepatitis virus as the source material obtained from two patients using a well defined, standardized 15-step scientifically acceptable method[6] (after a proper pathogenetic trial or proving). Hepatitis C virus is known to cause hepatitis, cirrhosis, malignancy, fibrotic changes, thrombocytopenia, hepatic portal hypertension, chronic organ inflammation, *etc*. It is the cause of 27% of cirrhosis cases and 25% of hepatocellular carcinoma worldwide[7]. In view of the ability of hepatitis virus to mainly target liver causing critical diseases like hepatitis, inflammation and cirrhosis of liver and malignancy, we were interested in examining if ultra-high dilutions (30C, diluted homeopathically 10^{-60} times) of the Hep C nosode (mother tincture) could demonstrate anticancer potential *in vitro* at the molecular level, as based on the homeopathic doctrine of "like cures like" through some scientifically accepted molecular biology protocols and also to test if it could modulate some molecular biomarkers for cancer like expression of telomerase and topoisomerase II (Top II) enzymes in a favourable way, which had not been studied so far.

Therefore, in this study, the main hypotheses to be tested included: (1) if Hep C 30 nosode has any anticancer effect primarily on the liver cancer cell line, HepG2, the better responding cell line, using WRL-68 (derived from normal hepatocytes) as the normal control and placebo 30 (succussed diluted solvent ethanol of Hep C 30) as the negative control; (2) to undertake a thorough study on HepG2, and track down the possible signaling pathway of its action *in vitro* on HepG2 cell line; (3) to demonstrate if it can favorably modulate expressions of specific cancer biomarkers like telomerase and Top II by deploying certain scientifically acceptable molecular biology protocols.

2 Materials and methods

2.1 Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), and penicillin, streptomycin and neomycin (PSN) were procured from HiMedia (India). Fetal bovine serum (FBS), trypsin and ethylene diamine tetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were procured from Tarson (India). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), acridine orange (AO)/ethidium bromide (EB), dichloro-dihydro-fluorescein diacetate (H2DCFDA), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and rhodamine 123 were purchased from Sigma (USA). Annexin V-fluorescein isothiocyanate (FITC) and primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (USA) and secondary antibodies were purchased from Sigma (USA).

2.2 Source of the drug and preparation procedure

The Hep C 30 nosode was developed in the laboratory by one of us (RS) after getting due clearance from an independent Institutional Ethics Committee, namely, Homeopathy India Private Limited, Mumbai, comprising members from various government-accredited universities and research institutes and duly recognized by the Indian Council of Medical Research, Government of India, using an accepted 15-step elaborate protocol standardized as per compliance with the Central Council for Research in Homoeopathy, Technical Committee, Government of India, and results of the pathogenetic (proving) trial published[1–3]. Briefly, two patients, separately screened with hepatitis C genotypes I and III respectively, were ruled out for possible co-infections such as HIV, hepatitis B, gonorrhea, syphilis, *etc*., after proper pathological and biochemical tests conducted by qualified and experienced pathologists and biochemists, respectively; blood was drawn, serum expression was determined, and serum filtration was carried out to remove large particles, possible bacteria and other cell debris by standard method. The primary source material was diluted up to 5C with sterile distilled water which was considered as the stock solution, from which the 30C potency was finally made by using 90% ethanol (made from high-quality laboratory

grade absolute alcohol) at each step of potentization as per the homeopathic procedure of succussions and dilution from 6C onward using a standard electromechanical potentizer[3]. Further, safety check (for possible human use) was subsequently carried out for Hep C nosode 30 C potency by reverse transcription-polymerase chain reaction method before use of the drug in the experiments. It was further established and documented that all the samples of hepatitis C virus nosode 30 C were tested negative for live hepatitis C virus. The intellectual property right for the preparation of nosode Hep C 30 has now been patented (IPR 240617, New Delhi, India). Placebo 30C was prepared in the same way by the same method of potentization using the same stock of 90% ethanol at each step of potentization, but without adding the starting serum material (drug) that was used only for the verum Hep C 30 preparation[6].

2.3 Choice of cell line for preliminary and elaborate studies

With the primary objective to assess its possible anticancer effects, preliminary studies were conducted to examine if Hep C 30 had any cytotoxic effect affecting cell viability in three types of cancer cells, HepG2 (liver cancer), A549 (lung cancer) and MCF-7 (breast cancer) by MTT assay, DAPI staining and cellular morphology analysis. We used results of these studies to select one cell line that showed the best response to the drug, for carrying out further elaborate studies deploying several other pertinent protocols.

2.4 Cell culture

Liver cancer cell line (HepG2), breast cancer cell line (MCF-7) and lung cancer cell line (A549) were procured from the National Centre for Cell Science (NCCS), Pune, India. WRL-68 cells were also collected from the NCCS, as certified derivatives of normal liver hepatocytes. The cells were maintained in a humidified incubator (ESCO, Singapore) with ambient oxygen and 5% carbon dioxide at 37 °C. Cells were cultured in DMEM with 10% heat-inactivated FBS and 1% PSN. Cells harvested with 0.025% trypsin-EDTA in phosphate-buffered saline (PBS) were plated at required cell numbers and allowed to adhere for required time before treatment.

2.5 MTT assay

A549, MCF-7, HepG2 and WRL-68 cells were dispensed into 96-well flat bottom micro-titer plates at a density of 1×10^3 cells per well. Different cells were treated with various concentrations of Hep C 30 (0.5 μL to 5 μL per 100 μL media) and incubated for 24 h. MTT solution (10 μmol/L) was then added to each well and the cells were incubated for 3 h at 37 °C. Formazan crystals formed were dissolved in 100 μL acidic isopropanol and the optical density (OD) was measured at 595 nm in an enzyme-linked immunosorbent assay reader (Thermo Scientific, USA)[4]. Although in MTT assay and other preliminary tests like DNA fragmentation assay and cellular morphology assay, Hep C 30 showed apoptotic effects on A459 and MCF-7 cells in varying degrees, in view of greater cytotoxic effects against HepG2 cells, this cell line was selected for further detailed experimental works, since we were more interested in assessing its effect on liver hepatocytes. WRL-68 cells served as one arm of the control.

2.6 Selection of dose

The selection of dose of the drug-treated as well as placebo-treated series was made through a range-finding trial. HepG2 cells were treated with Hep C 30 (0.5 μL to 5 μL per 100 μL media) and the corresponding doses of placebo, and MTT assay was conducted; range-finding trial results were used to find out the optimum (lethal dose 50, LD_{50}) dose and two other suitable drug doses below LD_{50}. Similarly, MTT assay was also carried out with the normal cell lines (WRL-68) treated with Hep C 30 and placebo 30 with a similar objective.

2.7 Cell morphological analysis

Morphology of HepG2 cells treated for 24 h with the three different doses of Hep C 30 separately was studied along with a control group exposed to only 2.7 μL 90% succussed ethanol (placebo) per 100 μL of media, the highest placebo dose corresponding to the highest drug dose. Cells were observed under an inverted phase-contrast microscope (DMi1 Leica, Germany), equipped with a digital camera.

2.8 Apoptotic analysis

After treatment with the three doses of Hep C 30 (2%, 2.5% and 2.7% media, respectively) and the placebo separately, HepG2 cells were washed with PBS and fixed in chilled 70% ethanol. After fixation, cells were treated with RNase (5 mmol/L) and incubated for 10–15 min in the dark at 37 °C. Subsequently, cells were stained with annexin V and PI as described by Matassov *et al*[8]. The 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.

2.9 AO/EB staining and DAPI staining for nuclear morphology analysis

One control (2.7% media) and three drug doses (2%, 2.5% and 2.7% media of Hep C 30) were selected for this experiment. After 24-hour incubation, cells were fixed with 2% paraformaldehyde. Then the cells were stained with AO/EB (1 mg/mL) and DAPI at 10 μmol/L concentration and observed under a fluorescence microscope (Leica DFC365 FX, Germany).

2.10 Analysis of Top II activity by confocal microscopy

Hep C 30-treated cells (2%, 2.5% and 2.7% media)

as well as 2.7 μL placebo per 100 μL media were incubated for 24 h with primary antibody of Top II (Santa Cruz Biotechnology, Inc., USA) at 4 °C overnight and developed with secondary FITC-conjugated anti-cytochrome *c* antibody. Then the cells were stained with DAPI at 10 μmol/L concentration and photographs were taken under a confocal microscope (Carl Zeiss LSM 510 META Laser Scanning Microscope).

2.11 DNA fragmentation assay

Control- and drug-treated HepG2 cells (2%, 2.5% and 2.7% media of Hep C 30) were washed in PBS and incubated with DNA lysis buffer (10 mmol/L Tris, 400 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.2 mg/mL RNase and 0.1 mg/mL proteinase K overnight, and then centrifuged at 1 200×*g* at 4 °C. Supernatants were then mixed with phenol-chloroform-isoamyle mixture (25:24:1) and the bi-layered mixture was centrifuged at 1 500×*g* at 4 °C for 15 min. DNA was then precipitated from the aqueous layer using 100% ethanol, and re-dissolved in 20 μL of Tris-EDTA buffer (10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA). The extracted DNA was further purified using proteinase K and RNase to remove contaminating proteins and RNAs, respectively. Purified DNA was separated using 1.5% agarose gel electrophoresis and bands were visualized under an ultraviolet trans-illuminator, followed by digital photography.

2.12 Analysis of changes in mitochondrial membrane potentials

After a 24-hour incubation, control and treated (2%, 2.5% and 2.7% media of Hep C 30) cells were fixed with 2% paraformaldehyde and then incubated with 10 μmol/L rhodamine 123 for 30 min at 37 °C in the dark. After incubation, cells were immediately analyzed using fluorescence microscopy (Leica, Germany).

2.13 Determination of reactive oxygen species accumulation

Reactive oxygen species (ROS) accumulation was assayed qualitatively at 24 h incubation after Hep C 30 treatment. The cells were fixed with 70% chilled ethanol and then incubated with 10 μmol/L H2DCFDA for 30 min at 20–25 °C in the dark. Fluorescence intensity was measured by fluorescence microscopy (Leica, Germany).

2.14 Western blot analysis

Drug- and placebo-treated HepG2 cells were seeded into 75 mm plates (Tarson, India) at a density of 1×10^5 cells per well. Cells were treated with different concentrations of Hep C 30 separately and incubated for 24 h. An equal amount of protein (50 μg) was run on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinyl difluoride membrane. After 3% bovine serum albumin blocking, the membranes were incubated overnight, at 4 °C, with specific primary antibodies, including p53, TERT, Bcl-2, Bax, caspase-3, cytochrome *c* or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The membrane was then incubated for 2 h with alkaline phosphatase-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.

2.15 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.

3 Results

3.1 Cell viability assay

Three different doses were selected based on the results of the range-finding trials of MTT assay, indicating the LD_{50} as 2.7% media; this LD_{50} dose and two doses below the LD_{50} (2.5% media and 2% media) were selected for further experimentations. Control cells were initially treated with 2.0, 2.5 and 2.7 μL succussed ethanol (ethanol 30 C) prepared from the same stock with which the drug was prepared per 100 μL media, but there being virtually no difference in result among these doses, also, only the highest of placebo dose, that is 2.7 μL of succussed ethanol was considered as the vehicle control for all the subsequent experiments.

Cell viability was primarily assessed through MTT assay with different cancer and normal cell lines. Hep C 30 reduced the viability of MCF-7, HepG2 and A549 cells with different doses. In 24-hour incubations, 50% cell death occurred at the dose of 3.91, 2.71 and 3.041 μL per 100 μL media on MCF-7, HepG2 and A549 cell lines, respectively (Figure 1). However, the cytotoxicity was found to be significantly greater in HepG2 cells. The cytotoxicity was increased in HepG2 cells in a dose-dependent manner like that of other cell lines used. Experimental results for the normal cell line (WRL-68) revealed that there was very less cytotoxicity produced by Hep C 30 after 24 h of treatment. In case of WRL-68, the percentage of viability was actually reduced by 21.7% (100%−78.3%) at the highest dose of Hep C 30 (5% media) for 24 h, as against 63.4% (100%−36.4%) in MCF-7, 80.83% (100%−19.17%) in HepG2 and 79.25% (100%−20.75%) in A549 cells.

3.2 Morphological changes in drug-treated HepG2 cells

Experimental results showed that morphological changes were observed in HepG2 cells treated with Hep C 30. Rounding off of the cytoplasmic periphery along with gradual detachment of cells from the substrate was observed. Shrinkage of cell and blebbing of cell membrane were found in Hep C 30-treated cells (Figure 2). In the placebo-control, these changes were insignificant.

3.3 Apoptosis analysis by FACS

Apoptosis was measured by annexin V/PI staining with the externalization of phosphatidyl serine. Cells showed distinct positive binding with annexin V when treated with Hep C 30, indicating the movement of phosphatidyl serine to the outer cell surface. Treatment with Hep C 30 caused significant apoptotic cell death in HepG2 cells (Figure 3), not observed in the placebo control.

3.4 Nuclear changes induced in Hep C 30-treated cells

The results of DNA fragmentation assay showed that Hep C 30 treatment induced cellular DNA fragmentation leading to DNA damage as observed from different fragmented bands of DNA incubated with Hep C 30 as compared to the control. Experimental analysis also suggested initiation of cell DNA fragmentation in Hep C 30-treated groups (Figure 4).

AO/EB staining result showed a change in the

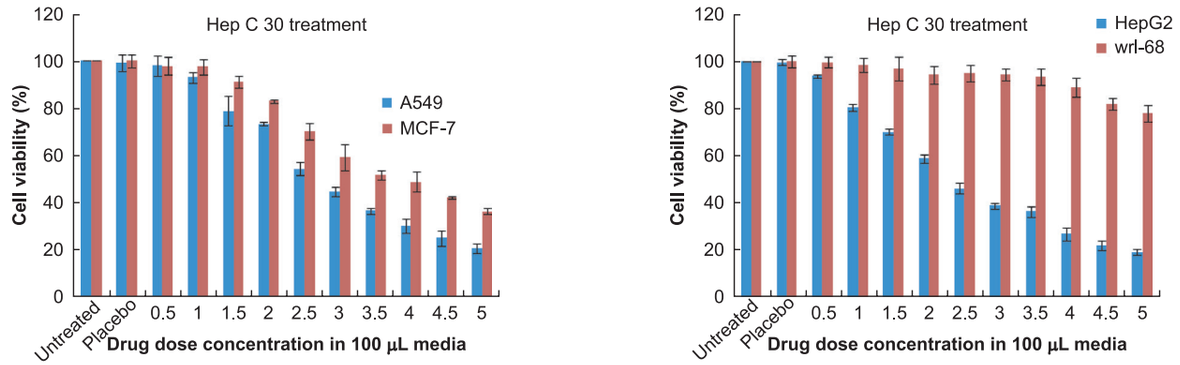

Figure 1 Cell viability assay

Cells (MCF-7, HepG2, A549 and WRL-68) were treated with 0.5 µL to 5 µL per 100 µL media of Hep C 30 for 24 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results showed gradual reduction in the viability of cell lines MCF-7, HepG2 and A549. Hep C 30 produced negligible cytotoxicity in normal cell line (WRL-68) after 24 h of treatment. Data are presented as mean ± standard error of mean.

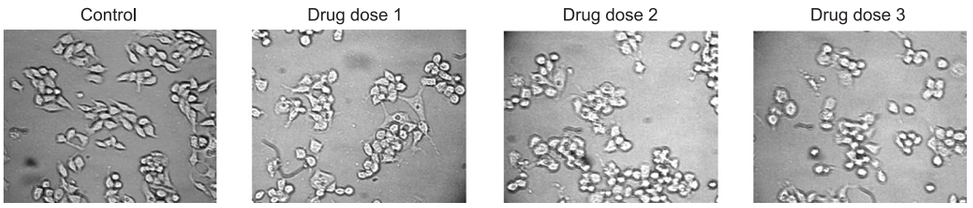

Figure 2 Morphological analysis

HepG2 cells showed membrane blebbing, cell periphery shrinkage and rounding off of cells in treatment group.

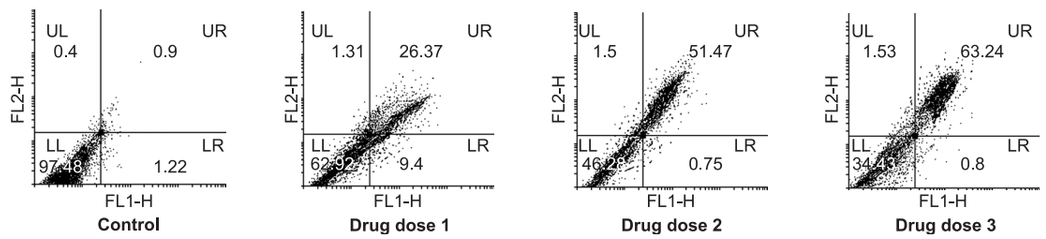

Figure 3 Annexin V/propidium iodide assay

HepG2 cells were undergoing apoptosis after Hep C 30 treatment. Dot plot suggested that more number of apoptosis occurred at the highest dose of Hep C 30 (2.7% media) after 24 h incubation (upper left = dead cells, lower left = live cells, upper right = late apoptotic cells and lower right = early apoptotic cells).

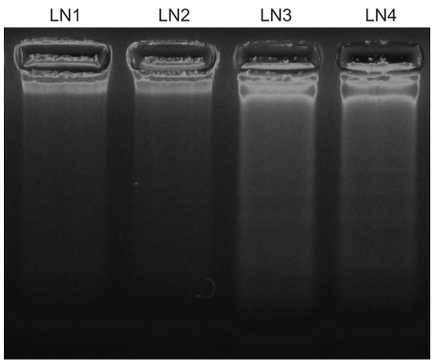

Figure 4 DNA fragmentation assay
Results indicated that DNA-fragmentation was present in Hep C 30-treated cells (LN1: Control; LN2: Drug dose 1; LN3: Drug dose 2; LN4: Drug dose 3). Fragmentation was prominently noticeable at the drug doses 2 and 3.

fluorescence pattern from green (normal cellular DNA) to orange (nicked cellular DNA). Increased fluorescence of EB in drug-treated cells revealed that chromosomal condensation occurred due to Hep C 30 treatment which was another sign of apoptosis (Figure 5A).

After DAPI staining (Figure 5B), nuclear condensation was observed in HepG2 cells treated with the highest level of Hep C 30 (2.7% media) as compared with the control. This result suggested that Hep C 30 could potentially initiate the process of cellular DNA damage by commencing with the nuclear condensation process.

Decreased levels of Top II in the cellular nucleus were shown by confocal microscopy. Experimental results indicated that Top II expression was decreased in the nuclei of cells in the drug-treated groups (2%, 2.5% and 2.7% media), when compared with the control (Figure 6).

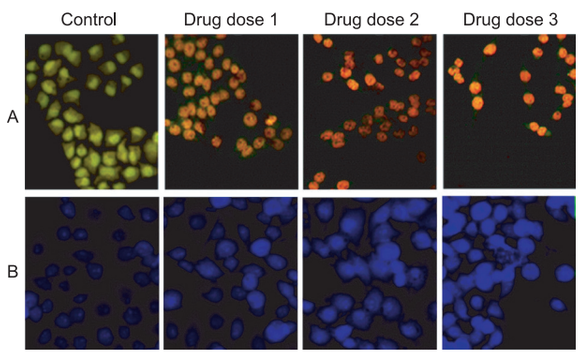

Figure 5 Analysis of nuclear morphology by (A) acridine orange/ethidium bromide staining and (B) 4',6-diamidino-2-phenylindole staining
Both staining images have shown changes of nuclear morphology after Hep C 30 treatment. Highest chromatin condensation was observed in cells treated with the highest dose (2.7% media) of Hep C 30 treatment, as indicated by brighter flurorescence intensity (40×).

3.5 Depolarization of mitochondrial membrane potentials

The rhodamine staining assay revealed that there was a decrease in mitochondrial membrane polarization (MMP) after Hep C 30 treatment. The reduction of MMP appeared to be dose-dependent in nature and maximum depolarization was observed with 2.7% media of Hep C 30 treatment (Figure 7), not observed in the control.

3.6 ROS generation

The fluorescence images revealed that Hep C 30 treatment initiated accumulation of ROS in HepG2 cells, which was not found in the control. ROS generation gradually increased with an increase in drug dose (Figure 8).

3.7 Change in expressions of different signal proteins related to apoptosis

After Hep C 30 treatment, expression of proteins related to cell survival and apoptosis in HepG2 cells was quantified. The expression of p53 and Bax was up-regulated after Hep C 30 treatment. On the other hand, the expressions of TERT and Bcl-2 decreased upon treatment of Hep C 30. This probably resulted in the release of cytochrome *c* and other pro-apoptotic factors from the mitochondria leading to activation of caspases. The signaling cascade would thus indicate the induction of apoptosis in Hep C 30-treated HepG2 cells, not observed in the control (Figure 9).

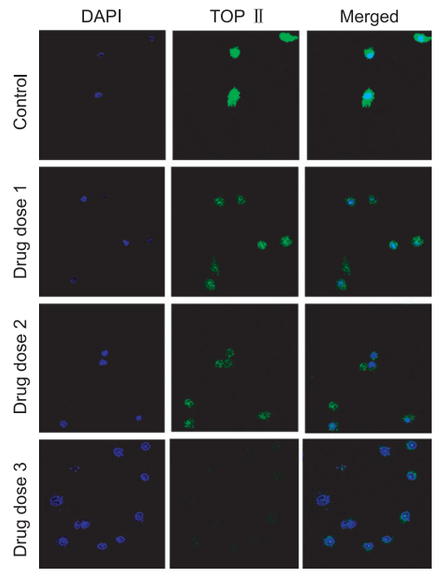

Figure 6 Confocal microscopic analysis under 40× magnification
Expression of TOP II in nucleus in cells was studied after treatment with Hep C 30. Changes of expression of Top II were observed in treated group as compared to control. DAPI: 4',6-diamidino-2-phenylindole dihydrochloride; Top II: topoisomerase II.

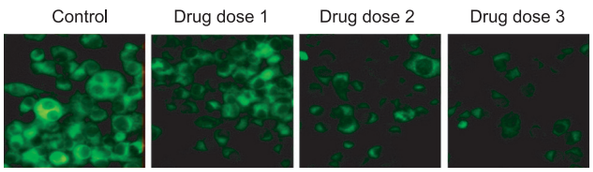

Figure 7 Depolarization of MMP seen under fluorescence microscopy (40×)
Fluorescence intensity was reduced gradually up to 59.337% at increasing Hep C 30 doses, indicating gradual reduction of MMP. MMP: mitochondrial membrane potential.

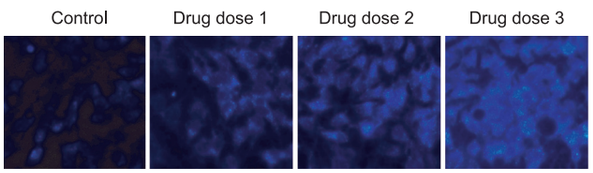

Figure 8 Reactive oxygen species accumulation
Accumulation of reactive oxygen species was increased with dose-dependent manner after Hep C 30 treatment.

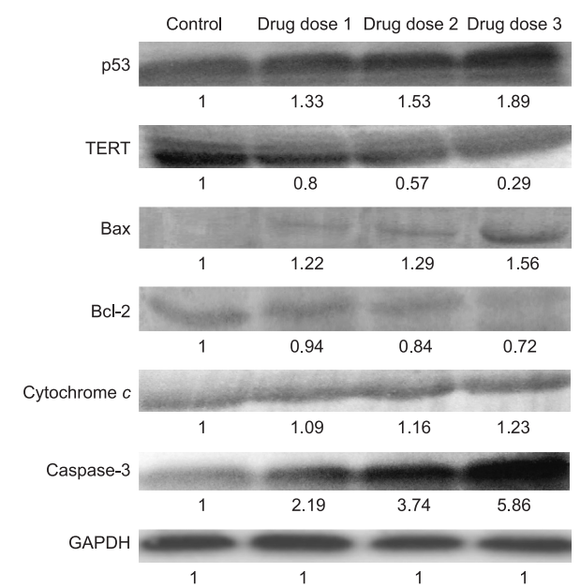

Figure 9 Analytical data and images of Western blots
p53, Bax, cytochrome *c*, and caspase-3 activities were up-regulated and TERT and Bcl-2 expressions were down-regulated by Hep C 30 treatment for 24 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the loading control.

4 Discussion

Results of this study revealed that Hep C 30 had ability to modulate telomerase and Top II enzyme activities in a favorable way that is expected of an agent having anticancer potential. This finding is highly significant as both these enzymes are actively associated with the divisional activities of cells and DNA and cancer is a disease of uncontrolled cell division requiring an active synthesis of telomeres and DNA. These two enzymes are considered as strong biomarkers for cancer development and growth, and are therefore considered as important targets of anticancer agents. The ability of this ultra-highly diluted drug to have positive influence on these two enzymes is therefore very significant findings, and not reported by any earlier worker. This finding also gives credence to the gene-regulatory hypothesis proposed by Khuda-Bukhsh[9,10] to explain molecular mechanism of biological action of potentized homeopathic drugs, particularly those diluted above Avogadro's limit.

Human somatic cells are known to grow by mitosis and almost all cells are destined to die by apoptosis, an intrinsic process of programmed cell death otherwise termed as the physiological process of driving cells to commit suicide. Cancers can occur when this balance or control of cellular growth and apoptosis is disturbed, either by an uncontrolled increase in cell proliferation or a decrease in cell death[11]. Therefore, one of the goals of effective cancer therapy is to promote the death of cancer cells without causing too much damage to normal cells. Thus, most cancer drugs are strategically targeted towards working in two ways, by induction of apoptosis as well as by inducing direct cytotoxicity. In many cases, the otherwise effective orthodox anticancer drugs soon face resistance from cancer cells as they start to skip signals for apoptosis which makes these drugs quite ineffective[11,12]. Further, most anticancer orthodox drugs routinely used to treat cancer also have toxic effects on normal cells, an undesirable feature that often forces them to be withdrawn prematurely. Therefore, such drugs are highly sought after for therapeutic use in oncology when they have preferential cytotoxic action against cancer cells, but without causing any significant cytotoxicity in the normal cells. Results of this study showed that the nosode C 30 appeared to promote apoptosis in cancer cells but with only very insignificant apoptotic effect causing concurrent death of normal cells.

The findings of this study revealed further by cytotoxicity assay that Hep C 30 induced cytotoxic effect in varying degrees on all three cancer cells tested; it also produced much less cytotoxicity to the normal hepatocyte cell line WRL-68. It was further revealed on comparison of effects of LD_{50} dose of Hep C 30 on these three cell lines that the drug induced best effect on HepG2 showing its effect in a dose-dependent manner. Since hepatitis C virus often causes cirrhosis of liver or liver cancer, our present findings tend to support that the nosode derived from hepatitis C virus appeared to have a more prominent and directed action in liver cancer cells than in the cancer cells of lung and breast origin.

It is an accepted fact that biochemical markers of apoptosis include exposure of phosphatidylserine on the cell surface[13]. This was also confirmed in this study as annexin V/FITC assay demonstrated that Hep C treatment induced apoptosis with externalization of phosphatidyl serine on the cell surface.

DNA has now been established to be the molecular target for many of the drugs that are used in cancer therapeutics. There is an intrinsic and accurate repair mechanism in living systems for early repair of any damaged DNA with double-strand breaks and it is considered essential for maintenance of normal metabolic processes of life. Indeed, defective DNA double-strand breaks and failure of their repair can lead to toxicity and large-scale sequence rearrangements that cause cancer and promote premature aging[14,15]. Thus, gaining knowledge about whether the drug has the ability to get transported into the nucleus of the cancer cells becomes important. Results of nuclear condensation assay of the cancer cells after administration of the drug in different doses showed that the drug could effectively enter nuclei, and that made us inclined to investigate further to verify whether the drug could potentially bind with DNA. The DNA fragmentation assay also corroborated these findings, indicating a positive relationship to exist between the drug dose and rate of DNA damage in cancer cells. This result in turn led us to further investigate if there was any modulation of p53 signal protein in cancer cells after administration of the drug. Up-regulation of p53 protein was observed, which would clearly point out that the drug actually caused damage to the DNA[16,17] as revealed from the results obtained in Top II. Top II constitutes a family of nuclear enzymes essential for proper replication process of DNA in all living cells. These enzymes are capable of transferring one double helix through a transient break in another DNA double helix. Top II plays important roles in DNA metabolic processes, in which they are involved in DNA replication, transcription, chromosome condensation and de-condensation[18]. Top II has also been reported to be the cellular target for a number of widely used anticancer agents that are currently in clinical use[19,20]. Down-regulation of Top II expression in cancer cells after Hep C 30 treatment indicated that this drug acted as a topoisomerase inhibitor that contributes to the cancer cells to be led towards death.

Many anticancer drugs are known to act through generation of ROS, that in turn induces apoptosis. Some research suggests that cancer cells have increased level of ROS with an altered redox status, relative to normal cells[21]. Therefore, it becomes necessary to look for such other possible factors associated with the phenomenon of apoptosis. Change in MMP is as one such phenomenon directly related to the drug-induced modulation of ROS production. The present work revealed that treatment with Hep C 30 resulted in a considerable increase in ROS generation and corresponding decrease in MMP, corroborating with some earlier research findings on anticancer drugs[22].

Telomerase is commonly expressed more in human cancer cells, which is deeply associated with cell division, as telomere synthesis to a required extent is the key factor for cell division. Increased telomerase expression is a pre-requisite for growth of cancer cells, distinguishing them from other normal cells in the body where telomerase activity remains controlled. Recent studies[23] also suggest that telomerase is implicated in tumor progression in some unexpected ways. These observations actually stimulated us to focus our attention to pinpoint to investigate if Hep C 30 could inhibit telomerase activity efficiently that could hinder the uncontrolled cell division which is a hallmark of cancer. Indeed, the present findings revealed that Hep C 30 down-regulated expression of the telomerase reverse transcriptase which is the catalytic sub-unit of telomerase enzyme that also has a particular role in induction of apoptosis in cancer cells[24].

The under-expression of pro-apoptotic protein Bax and the over-expression of anti-apoptotic protein Bcl-2 would clearly indicate that there was a considerable change in the balance of these proteins' ratio, which is known to be in favor of triggering the apoptotic process[25].

To further test whether the apoptotic process was activated via the intrinsic mitochondrial pathway, we also investigated the status of cytochrome *c* release, associated with the changes in Bax/Bcl-2 protein ratio in drug-treated cancer cells. This phenomenon was further supported by caspase-3 activation indicated by the immunoblot studies. The activation of caspase-cascade clearly indicated that Hep C 30 acted by turning on the apoptotic intrinsic pathway.

Thus, overall results would clearly indicate that the drug not only has a positive role in breaking DNA double helix inducing DNA damage, but also simultaneously increases the rate of intracellular ROS, leading to the initiation of apoptosis through the mitochondria-mediated intrinsic pathway. Therefore, Hep C 30 nosode has a great potential for being used, at least as a supporting therapy in the treatment of severe liver ailments including cancer and a more thorough research study is warranted to learn more about the efficacy of Hep C 30 nosode in other diseases like cirrhosis and viral hepatitis with well designed scientific experiments. Further, micro-doses of Hep C 30 nosode at 30C potency is absolutely safe for human use as it is diluted 10^{60} times, and possibility of the presence of even a single molecule of the original serum material is highly improbable. But it also raises a concern as to what could be the basis of its medicinal

efficacy then. There has been a paradigm shift in recent years as several authors have demonstrated the presence of nanoparticles of the original drug substances in ultra-highly diluted homeopathic remedies, particularly derived from metal compounds like Cuprum met and Aurum met[26] and also in certain nosodes[27]. Incidentally significant physiological/enzymatic changes brought about by ultra-highly diluted remedies[28] and by nano-scale natural products have been well documented by Bell and her collaborators[29–31]. Therefore, it is most likely that the nanoparticles occurring in the Hep C nosode may have a major role in accomplishing its anticancer effects as revealed from results of the present study.

5 Acknowledgements

Part of the work was financed through a Senior Research Fellowship grant of the University Grants Commission, Government of India, sanctioned to JM, and part through Emeritus Fellowship from a grant provided to ARKB. Sincere thanks is due to Dr. Arindam Bhattacharyya, Associate Professor, Department of Zoology, Calcutta University, for his kind help in performing the confocal microscopy.

6 Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1 Shah R. Hepatitis C Nosode: the preparation and homeopathic pathogenetic trial. *Homeopathy*. 2013; 102(3): 207–214.
2 Shah R. Scientific method of preparing homoeopathic nosodes. *Indian J Res Homeopath*. 2014; 8(3): 166–173.
3 Shah R. HIV Nosode: The homeopathic pathogenetic trial. *Forsch Komplementmed*. 2015; 22(3): 156–162.
4 Shah R. A clinical evaluation of a Hepatitis C Nosode in the treatment of hepatitis C. *J Altern Complement Med*. 2016; 22(3): 197–203.
5 Mondal J, Samadder A, Khuda-Bukhsh AR. Psorinum 6× triggers apoptosis signals in human lung cancer cells. *J Integr Med*. 2016; 14(2): 143–153.
6 Alter MJ. Epidemiology of hepatitis C virus infection. *World J Gastroenterol*. 2007; 13(17): 2436–2441.
7 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; 65(1–2): 55–63.
8 Matassov D, Kagan T, Leblanc J, Sikorska M, Zakeri Z. Measurement of apoptosis by DNA fragmentation. *Methods Mol Biol*. 2004; 282: 1–17.
9 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(1): 43–46.
10 Khuda-Bukhsh AR. Current trends in ultra-high dilution research with particular reference to gene regulatory hypothesis: review article. *Nucleus*. 2014; 57(1): 3–17.
11 Gerl R, Vaux DL. Apoptosis in the development and treatment of cancer. *Carcinogenesis*. 2005; 26(2): 263–270.
12 Kaufmann SH, Vaux DL. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene*. 2003; 22(47): 7414–7430.
13 Krysko DV, Vanden Berghe T, Parthoens E, D'Herde K, Vandenabeele P. Methods for distinguishing apoptotic from necrotic cells and measuring their clearance. *Methods Enzymol*. 2008; 442: 307–341.
14 Jackson SP. Sensing and repairing DNA double-strand breaks. *Carcinogenesis*. 2002; 23(5): 687–696.
15 Abbotts R, Thompson N, Madhusudan S. DNA repair in cancer: emerging targets for personalized therapy. *Cancer Manag Res*. 2014; 6: 77–92.
16 Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. *Oncogene*. 1999; 18(53): 7644–7655.
17 Christmann M, Kaina B. Transcriptional regulation of human DNA repair genes following genotoxic stress: trigger mechanisms, inducible responses and genotoxic adaptation. *Nucleic Acids Res*. 2013; 41(18): 8403–8420.
18 Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol*. 2002; 3(6): 430–440.
19 Willmore E, de Caux S, Sunter NJ, Tilby MJ, Jackson GH, Austin CA, Durkacz BW. A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia. *Blood*. 2004; 103(12): 4659–4665.
20 Thakur DS. Topoisomerrase II inhibitors in cancer treatment. *Int J Pharm Sci Nanotechnol*. 2011; 3(4): 1173–1181.
21 Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov.* 2009; 8(7): 579–591.
22 Liu Y, Jiao R, Ma ZG, Liu W, Wu QQ, Yang Z, Li FF, Yuan Y, Bian ZY, Tang QZ. 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(3): 3400–3408.
23 Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science.* 1994; 266(5193): 2011–2015.
24 Shay JW, Zou Y, Hiyama E, Wright WE. Telomerase and cancer. *Hum Mol Genet*. 2001; 10(7): 677–685.
25 Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and molecular targeting therapy in cancer. *Biomed Res Int*. 2014; 2014: 150845.
26 Chikramane PS, Kalita D, Suresh AK, Kane SG, Bellare JR. Why extreme dilutions reach non-zero asymptotes: a nanoparticulate hypothesis based on froth flotation. *Langmuir*. 2012; 28(45): 15864–15875.
27 Rajendran ES. *Nanodynamics*. Cochin: Mohna Publications.

2015: 170–178.
28 Eskinazi D. Homeopathy re-revisited: is homeopathy compatible with biomedical observations? *Arch Intern Med.* 1999; 159(17): 1981–1987.
29 Bell IR, Schwartz GE, Boyer NN, Koithan M, Brooks AJ. Advances in integrative nanomedicine for improving infectious disease treatment in public health. *Eur J Integr Med*. 2013; 5(2): 126–140.
30 Bell IR, Sarter B, Koithan M, Banerji P, Banerji P, Jain S, Ives J. Integrative nanomedicine: treating cancer with nanoscale natural products. *Glob Adv Health Med*. 2014; 3(1): 36–53.
31 Bell IR, Ives JA, Jonas WB. Nonlinear effects of nanoparticles: biological variability from hormetic doses, small particle sizes, and dynamic adaptive interactions. *Dose Response*. 2013; 12(2): 202–232.