Original Research

Encapsulated plant extract (*Gelsemium sempervirens*) poly (lactide-co-glycolide) nanoparticles enhance cellular uptake and increase bioactivity *in vitro*

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Abstract

Ethanolic extract of *Gelsemium sempervirens* (family: Loganiaceae), henceforth to be called EEGS, is used in various traditional systems of medicine. In homeopathy, EEGS is known as mother tincture of *G. sempervirens*, which is generally used to treat pain and respiratory ailments. We demonstrated earlier anticancer activity of crude EEGS by *in vitro* studies on human HeLa cells. To test the hypothesis if nanoparticle-encapsulated extract (now onwards to be called NEEGS) could enhance cellular uptake and thereby improve bioactivity, we formulated nanoparticle encapsulation based on poly (lactide-co-glycolide) (PLGA) and confirmed encapsulation by scanning electron microscopy (SEM) and atomic force microscopy. EEGS was encapsulated with 81.6% efficiency in PLGA biodegradable nanoparticle formulation and F68 (polyoxyethylene-polyoxypropylene) was used as a stabilizer. Dynamic laser light scattering and SEM indicated a particle diameter of 122.6 nm. The zeta potential of the drug-loaded nanoparticles was −14.8 mV. NEEGS was characterized for their biological activities in a skin cancer cell line A375 *in vitro*. NEEGS exhibited relatively rapid (30 min) and more efficient cellular uptake than their un-encapsulated counterpart (45 min). Analysis of data of apoptosis study using Annexin V-FITC, terminal transferase dUTP nick end labeling assay and DNA ladder revealed that encapsulated EEGS was more potent than their un-encapsulated counterpart in inducing apoptosis of A375 cells. Reverse transcriptase-polymerase chain reaction data of survivin, cyclin-D1, caspase-3, PCNA and p53 also corroborated well to suggest greater potentials of NEEGS as anticancer agents.

Keywords: nanoparticle encapsulation, PLGA, *Gelsemium sempervirens*, biodegradable polymer, *in vitro*, A375 cell line, apoptosis

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Introduction

In recent years, the incidence and mortality of malignant melanoma has continued to rise. While the early stages of the disease can often be cured by appropriate therapy, advanced and metastasised malignant melanoma is characterized by a short median survival time and implies a poor prognosis. For this reason the treatment of advanced melanoma remains a challenge in dermato-oncology.[1] However, rapamycin and celecoxib, two drugs with low toxicity and potential anticancer activity, offered hope for palliative use in malignant melanoma.[2] Only a few attempts have so far been made to nano-encapsulate potential anticancer drugs with biodegradable polymers and to see if the strategy could be helpful in enhancing anticancer potentials.

Gelsemium sempervirens (family: Loganiaceae), commonly known as Yellow Jessamine, is a plant of southeastern part of North America, found generally along sea coasts in dry to wet woods, thickets and sands. This plant has numerous therapeutic uses, although extreme care should be taken as all parts of the plant are considered toxic and potentially fatal if ingested in large doses. Ethanolic extract of *G. sempervirens* (EEGS) is used as homeopathic mother tincture and also as an ingredient in some other form of traditional medicines (TMs). In homoeopathy, *G. sempervirens* is mainly used to treat pain, respiratory ailments and occasionally for diseases of viral origin.[3] Recently, we[4] demonstrated anticancer potentials of EEGS on *in vitro* human HeLa cells. However, to our knowledge no attempt had been made to formulate nanoparticle encapsulation of EEGS based on poly (lactide-co-glycolide) (PLGA) and to study the relative efficacy of PLGA encapsulated homeopathic mother tincture (NEEGS) and its un-encapsulated counterpart (EEGS) on cellular uptake and bioactivity *in vitro* on A375 cells (human malignant melanoma).

Therefore, in the present study the hypotheses to be tested were as follows:

(1) Whether EEGS could be properly encapsulated with a biodegradable, non-toxic polymer like PLGA, which could have easy entry into target cell;
(2) If encapsulation was possible, whether NEEGS would show better cellular uptake and thereby render greater bioavailability than EEGS;
(3) Whether NEEGS would be bestowed with greater anticancer potentials than their EEGS counterpart;
(4) Whether they would induce more apoptosis and have antiproliferative activity, as revealed from Annexin V-fluorescein isothiocyanate (FITC) staining and the terminal transferase dUTP nick end labeling (TUNEL) assay;
(5) Whether the reverse transcriptase-polymerase chain reaction (RT-PCR) data of cyclin-D1, survivin, p53, proliferating cell nuclear antigen (PCNA) and caspase-3 would corroborate the findings of the apoptosis assays and indicate signaling pathway;
(6) Finally, whether the nanoparticle encapsulated form could be suitably characterized for its dynamic laser light scattering (DLS), zeta potential, polydispersity index (PDI) and morphology of nanoparticles inside the cell using scanning electron microscopy (SEM) and atomic force microscopy (AFM).

Materials and methods

Source of the EEGS

A volume of 100 mL of the mother tincture of *G. sempervirens* (Boiron Laboratory lot TJ0071 65% ethanol) was initially evaporated using a rotary evaporator at 40°C and dried in a vacuum desiccator. This dried EEGS was used for the present experiment.

Formation of blank nanoparticles and drug-loaded nanoparticles

PLGA nanoparticles were prepared by solvent displacement technique.[5] Briefly, 50 mg PLGA (purchased from Sigma Chemical Co, St Louis, MO, USA) and 10 mg of dried drug were dissolved in 3 mL acetone. The organic phase mixture was added drop-wise (0.5 mL/min) into 20 mL aqueous solution containing stabilizer 1% (polyoxyethylene-polyoxypropylene) (F68; w/v), which was then stirred for 12 h for complete evaporation of the organic solvent. The redundant stabilizer was removed from the nanoparticles by centrifugation at 25,000 *g* and 4°C for 30 min (REMI C 24 centrifuge, REMI Instruments Limited, Mumbai, India). The pellet was re-suspended in Milli-Q water and washed three times. The obtained nanoparticle-loaded suspensions were stored at 4°C until further use. The actual final drug loading content was calculated to be 8.16 mg after nanoparticle encapsulation.

Reagents for cell culture

Dulbecco's modified Eagle's medium (DMEM); fetal bovine serum (FBS); penicillin, streptomycin, neomycin (PSN); trypsin and ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Bioscience (San Jose, CA, USA). All organic solvents used were of HPLC grade. Propidium iodide, MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide] and all other chemicals used were purchased from Sigma Chemical Co.

Characterization of nanoparticles

Surface morphology

The surface morphology of the formulated nanoparticle-loaded drug was measured by SEM (Hitachi S 500, Hitachi, Dallas, TX, USA) equipped with a 15 kV SE detector with a collector bias of 300 V. The lyophilized samples were spread over double-sided conductive tape (12 mm) fixed onto a metallic stud. Samples for AFM imaging were prepared by placing a drop of PLGA nanoparticle suspension on a freshly cleaved mica sheet and allowing it to dry in the air. AFM (Veeco di CP-11) imaging was performed using AFM in amplitude and tapping modes.

Particle size and PDI

Average particle size and PDI were determined by DLS (Malvern Instruments, Southborough, UK) equipped with vertically polarized light supplied by an argon-ion laser.

Surface charge

Zeta-potential of the nanoparticles was also determined by DLS (Malvern Instruments). All measurements were determined from mean values of three experiments.

Encapsulation efficiency

The encapsulation efficiency (E, %) of EEGS loaded in PLGA nanoparticles was determined as follows: the nanoparticles were separated from the un-entrapped free drug using a NANOSEP (100 kDa cut-off) membrane filter and the amount of free drug in the filtrate was measured using a spectrophotometer (SHIMADZU UV-1700). The E (%) was calculated by $E\ (\%) = ([\text{Drug}]\ \text{tot} - [\text{Drug}]\ \text{free})/[\text{Drug}]\ \text{tot} \times 100$.

Cellular uptake by fluorescence study

The cellular uptake of EEGS and NEEGS in A375 cells was analyzed. In brief, cells were washed twice with phosphate-buffered saline (PBS). The washed cells were re-suspended in media and then incubated with EEGS and NEEGS for different time periods. Cells were then examined under a fluorescence microscope (Carl Zeiss, Standort Göttingen, Germany), and images were captured using a Axioscope Plus 2 and analyzed by Image J software. The percentage of fluorescent cells in the population was measured using a flow cytometer (FACS, Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using cell Quest software that collected and utilized individual fluorescence values of 10,000 cells to give a mean value for each of six experiments.

Cell culture study

The A375 cell line obtained from National Centre for Cell Science, Pune was grown at 37°C in 5% carbon dioxide atmosphere in DMEM supplemented with 10% FBS and 1% antibiotic (PSN). For experimental studies, cells were grown to 80–90% confluence, harvested with ice-cold buffer saline (PBS), plated at desired density and allowed to re-equilibrate for 24 h before any treatment.

Treatment of drug and placebo (65% ethanol)

Different amounts of EEGS and NEEGS, and blank nanoparticles (PLGA), namely 20 $\mu g/10^5$ cells, 40 $\mu g/10^5$ cells, 80 $\mu g/10^5$ cells, 100 $\mu g/10^5$ cells, 160 $\mu g/10^5$ cells and 200 $\mu g/10^5$ cells were poured into different wells as in previous experiment in HeLa cells.[4] A well was also provided with the ethanol placebo (2 μL 65%) ('vehicle' of EEGS). Since NEEGS was dissolved in water, PLGA nanoparticles were also dissolved in Milli-Q water to serve as a blank control. After 24 h treatment, different experiments were performed. To test the effect, the same number of cells (10^5) was used for each experiment. On a separate experiment, EEGS or NEEGS showed no or negligible cytotoxic effect on normal skin cells of mice cultured *in vitro*.

MTT assay

The MTT [3(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay[6] was used to determine energetic cell metabolism by measuring the activity of one of the oxidative enzymes. The dye is reduced in mitochondria by succinic dehydrogenase to an insoluble violet formazon product. A375 cells were cultured for 24 h on 96-well microplates. The cells were incubated for 24 h with and without compounds on test. Then MTT were added and after two hours formazon crystals were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm using a enzyme-linked immunosorbent assay reader.

Apoptosis analysis through TUNEL assay

The DNA strand break analysis was performed by labeling with Br-dUTP following the method of Darzynkiewicz *et al.*[7]. Briefly, $1–2 \times 10^5$ cells were suspended in 0.5 mL PBS. This suspension was transferred with a Pasteur pipette into a 5 mL polypropylene tube containing 4.5 mL of ice cold 1% formaldehyde in PBS. Cell pellet was re-suspended in 100 μL of FITC (or Alexa Fluor 488)-conjugated anti-Br-dU mAb solution procured from Abcam (Cambridge, MA, USA) (1:1000). Cells were incubated at room temperature for one hour. One milliliter of propidium iodide staining solution was added. Cells were incubated for 30 min at room temperature or 20 min at 37°C in the dark; after incubation cells were analyzed by flow cytometry (Becton Dickinson) and photographs were taken under a fluorescence microscope.

Double labeling of cells with Annexin V-FITC and propidium iodide

Perturbation in the cellular membrane occurs during the early stages of apoptosis that leads to a redistribution of phosphatidylserine to the external side of the cell membrane. Annexin V selectively binds to phosphatidylserine and thus enables the use of a fluorescence-labeled annexin V to identify the cells undergoing apoptosis. Cells were also stained with propidium iodide to distinguish early apoptotic cells from necrotic cells. A total of 1×10^5 cells per sample were taken into small centrifuge tubes. Cells were spun at 1200 g for five minutes, after which the supernatant was discarded and pellets were washed with 500 μL binding buffer. Then cells were harvested at 1200 g for five minutes and the cell pellet was re-suspended in 80 μL binding buffer. A volume of 10 μL Annexin V-FITC and 10 μL of propidium iodide labeling solutions were added and the cell suspension was incubated for 15 min at room temperature in the dark. Then the solution was ready for analysis by flow cytometry.

DNA laddering

Cells were harvested after different treatments into extraction buffer (10 mmol/L Tris-HCl pH 7.4, containing 10 mmol/L NaCl, 20 mmol/L EDTA and 1% Triton X-100) after 24 h of treatment. Genomic DNA was isolated by digesting the cell extract with 10 μg/mL of proteinase K at 56°C for 8–12 h. DNA was purified by phenol/chloroform precipitated with ethanol and dissolved in TE. Integrity of DNA was analyzed by gel electrophoresis on 1% agarose gels followed by ethidium bromide staining.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from the A375 cells using Trizol reagent according to the manufacturer's instructions, and the RNA concentration was determined spectrophotometrically at 260 nm. RNA was diluted to 2 μg/mL with water pretreated with diethylpyrocarbonate, containing 1U/μL RNase inhibitor. The following ingredients were placed into a tube: 1 μL RNA, 1 μL oligo(dT) 18, 1 μL reverse transcriptase, 2 μL 10 mmol/L deoxynucleoside triphosphate, 4 μL 5× buffer, and sterilized in distilled water up to a total volume of 20 μL. The mixture was incubated at 37°C for 60 min. After reverse transcription, the sample was heated at 95°C for five minutes to denature the reverse transcriptase, and then stored at −20°C for PCR.

The synthetic oligonucleotide primers used for RT-PCR (Table 1) were procured from Bangalore Genei (Bangalore, India) and Bioserve Biotechnologies India Pvt Ltd (Hyderabad, India). β-Actin was used as internal standard to normalize all samples for potential variations in mRNA content. Following PCR, 5 μL samples aliquots were subjected to electrophoresis on 1% (w/v) agarose gel for 20–30 min and then stained with ethidium bromide and photographed. Densitometry was performed using Total Lab software.

Statistical method

All results are expressed as mean ± standard error. Differences between the data of suitable control and EEGS, control and NEEGS, and between EEGS and NEEGS were calculated separately for analysis of statistical significance by following the Student's *t*-test method. *P* values less than 0.05–0.001 were considered significant. Homogeneity of the

Table 1 Primer sequences of cancer-related genes (human origin) used in this study

Primer name	Primer sequences
β-Actin	Catalogue number-117816,GeNei™ (purchased from Bangalore GeNei)
Cyclin-D1	F: GCGAGATGAGGCGATGGGGC R: CCTTCAGGGCGGCTGTGGTG
Survivin	F: ATGACGACCCCATGCAAA R: AGGATTTAGGCCACTGCCTT
PCNA	Catalogue number-117813,GeNei™ (purchased from Bangalore GeNei)
Caspase-3	F: AGGCGGTTGTAGAAGTTAATAAAGGT R: AGCGACTGGATGAACCAGGA
P53	Catalogue number-117810,GeNei™ (purchased from Bangalore GeNei)

different series was further tested by *post hoc* analysis followed by the Tukey one-way analysis of variance using SPSS 11.0 version and to demonstrate significant statistical differences ($P < 0.05$) (see Supplementary Tables), if any.

Results

Characterization of PLGA-encapsulated drugs

Surface morphology

The structure of the nanoparticles plays an important role in determining their adhesion to and interaction with cells. The features of morphology of PLGA-encapsulated drug under scanning microscopy and AFM with corresponding 3-D image are shown in Figures 1a–c. Figure 1a displays a spherical shape of nanoparticles with a smooth surface while AFM image of NEEGS (Figures 1b and c) shows the smooth surface of nanoparticles without any noticeable pinholes or cracks.

Particle size and PDI

It was reported in literature that smaller nanoparticles would have greater ease of entry and durability in the tumors.[8] It was suggested that large particles (<5 μm) would be taken up via the lymphatics and small particles (<500 nm) can cross the membrane of epithelial cells through endocytosis.[9] DLS data showed that the mean diameter of PLGA-encapsulated nanoparticles was 122.6 nm with PDI of 0.243, and represented in Table 2 and Figure 2.

Surface charge

Zeta potential is one of the most important physico-chemical characteristics of nanoparticles.[10] In the present study, we found that zeta potential of the drug-encapsulated form was −14.8 mV (Figure 2, Table 2). High absolute value of zeta potential indicates high electrical charge on the surface of the nanoparticles, which can cause strong repellent forces among particles to prevent aggregation of nanoparticles.

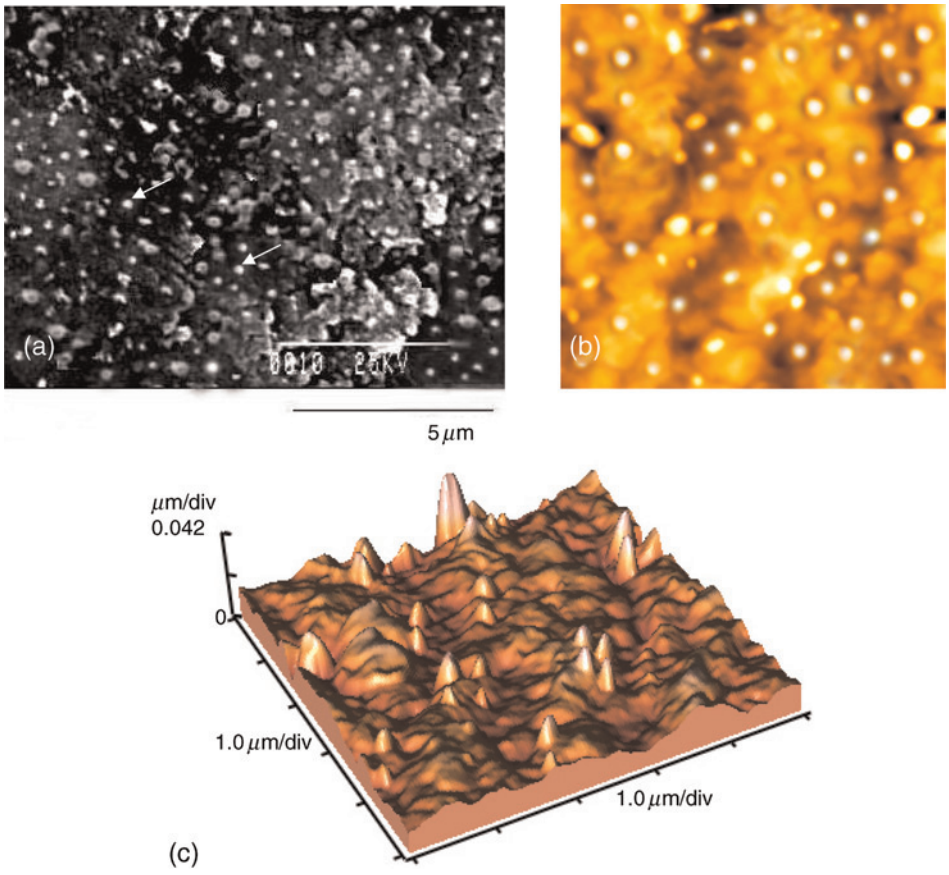

Figure 1 (a) SEM image of NEEGS. Arrows indicate nanoparticles. (b, c) Surface topography of nanoparticles obtained by AFM; (b) = 2D and (c) = 3D images. SEM, scanning electron microscopy; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*; AFM, atomic force microscopy (A color version of this figure is available in the online journal)

Table 2 Physical characterization of PLGA-encapsulated nanoparticles

Nanoparticle type	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
Nano-encapsulated ethanolic extract of *Gelsemium sempervirens*	122.6 ± 5	0.243 ± 0.021	−14.8 ± 1.5	81.6 ± 1.2

An increase in the negative surface charge of the PLGA nanoparticles is reported to decrease their size.

Encapsulation efficiency

PLGA-associated drug nanoparticles prepared by solvent displacement technique could achieve higher encapsulation efficiency with 81.6%.

Time of cellular entry

To check if the drug-loaded nanoparticles were internalized in tumor cells, the cellular uptake of NEEGS was evaluated on A375 cells at different time intervals. Cell line experiments are preferred as an initial study to provide preliminary information that helps to predict whether nanoparticles are good for drug release. Utilizing the fluorescence property of an alkaloid (scopoletin) of *G. sempervirens*,[4,11] cellular uptake of EEGS as well as NEEGS has been confirmed through fluorescence microscopic observation. The data of the time taken by EEGS and NEEGS have been presented in Figure 3. The data would indicate that the rate of entry into the cells started increasing after 30 min onwards for NEEGS and 45 min for EEGS. Therefore, NEEGS entered more rapidly than EEGS. Cells became saturated after 240 min in both capsulated and un-encapsulated forms. The percentages of NEEGS-treated cells (FACS analysis) that showed a statistically significant increase of fluorescence intensity ($P < 0.001$, $P < 0.01$) compared with EEGS-treated cells in different time intervals have been provided in Figure 4. In all, 76.2–80.3% cells showed fluorescence after 2–3 h incubation with NEEGS.

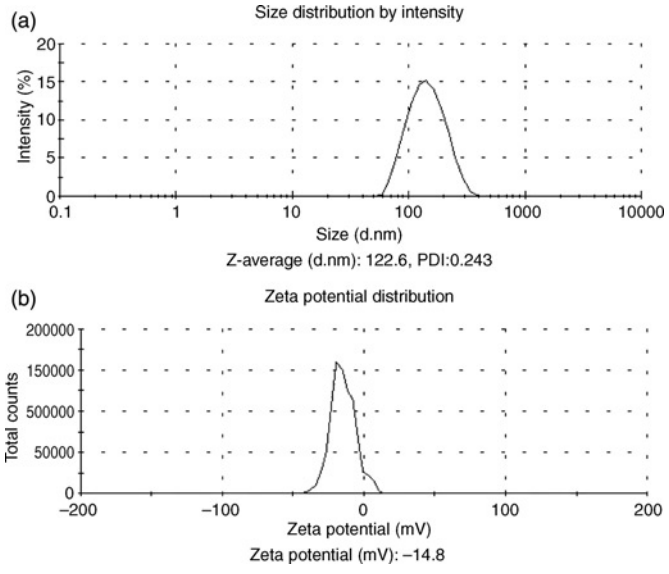

Figure 2 (a) Average particle size obtained from DLS data for NEEGS. (b) Zeta potential of NEEGS. DLS, dynamic laser light scattering; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*

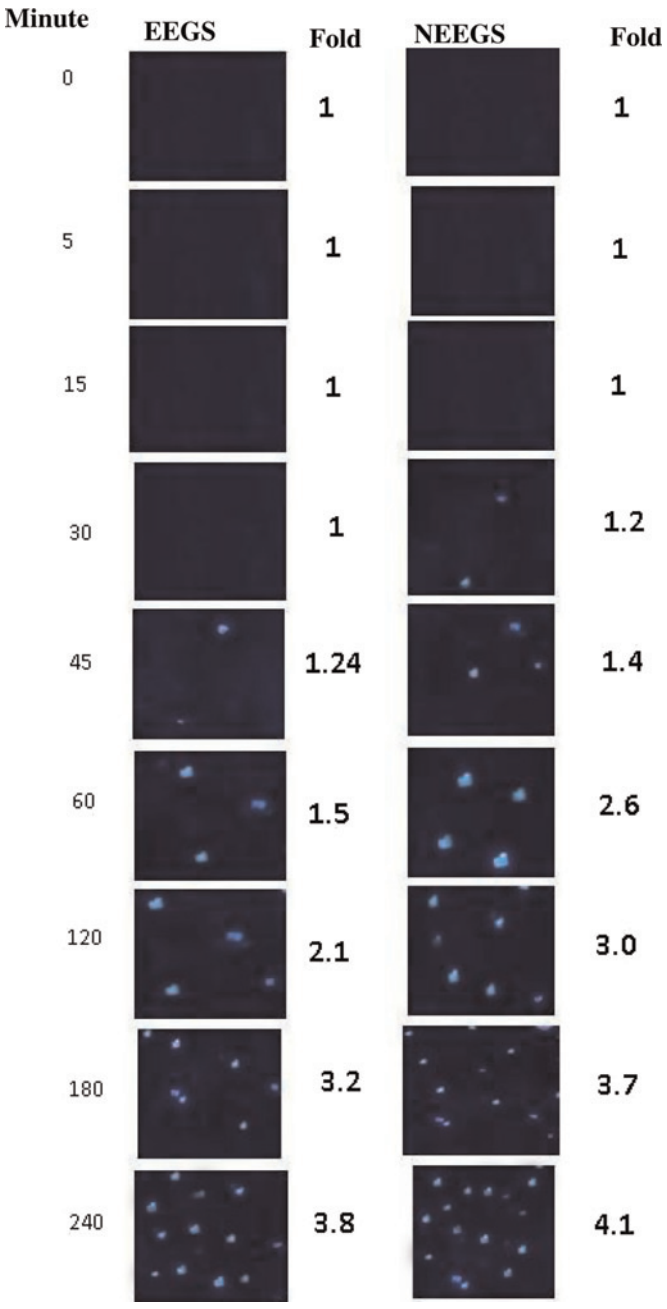

Figure 3 Cellular uptake of EEGS and NEEGS in A375 cells. The A375 cells were harvested at different time intervals and the cellular uptake (blue fluorescence) was monitored. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens* (A color version of this figure is available in the online journal)

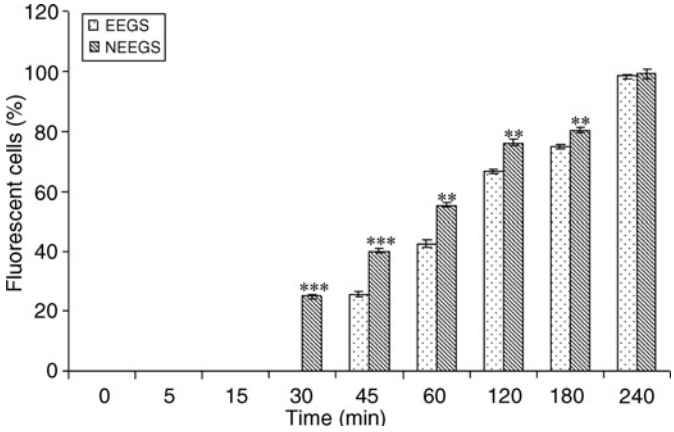

Figure 4 Percentages of cells exhibiting fluorescence at different exposures for EEGS and NEEGS, respectively. $^{***}(P < 0.001)$, $^{**}(P < 0.01)$. The data were taken from six replicates. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*

Percentage of cell viability of A375 cells

In order to understand the effect on cell viability, A375 cells were treated with either EEGS or NEEGS and MTT assay was performed. While no cytotoxic activity was observed for the drug-free nanoparticles, an inhibition on cell viability and growth was recorded in A375 cells exposed to EEGS and NEEGS. The results on percentage of cell viability as a result of treatment with different non-cytotoxic doses (20, 40, 80, 100, 160 and 200 μg) of both EEGS and NEEGS at 24 h have been furnished in Figure 5a. It would be evident from the data that, as compared with their respective controls, the percentages of cell viability were dramatically reduced ($P < 0.001$) in both EEGS and NEEGS. There was a significant difference ($P < 0.05$ or 0.01) in the degree of inhibition of growth of the A375 cells by NEEGS treatment at all doses as compared with that of EEGS (Figure 5b). The effect therefore was relatively strong in NEEGS than in EEGS.

Apoptosis assay by TUNEL assay using FACS

To examine if NEEGS modified the percentage of apoptosis, the TUNEL assay was conducted. Results of the TUNEL assay by FACS for determining the percentages of early and late apoptotic cells have been furnished in Figures 6a–c. NEEGS showed significantly ($P < 0.01$ through $P < 0.001$) greater apoptotic activities than EEGS. The frequencies of early apoptotic cells were 2.31% and 10.77% at 100 and 200 μg doses, respectively, for EEGS, while these were 3.18% and 20.66%, respectively, for NEEGS. The late apoptotic cells in EEGS comprised 10.35% and 19.05%, respectively, for 100 and 200 μg, while in NEEGS, these were 25.16% and 23.62%, respectively. Similarly, the microscopic data of TUNEL-positive cells (Figures 7a and b) were also in conformity with the FACS data of the TUNEL assay. In all, 52.3% cells were TUNEL positive for the 200 μg NEEGS-treated group as compared with 33.3% cells of 200 μg EEGS-treated group ($P < 0.05$, $P < 0.01$) (Figure 7b).

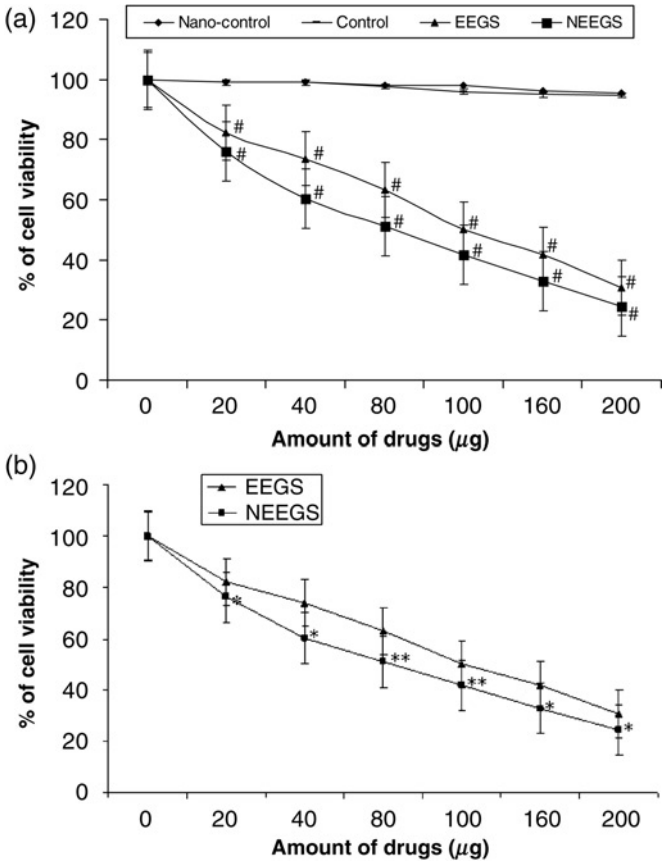

Figure 5 (a) Cell viability of A375 cells exposed to different amounts of blank nano-particles, EEGS and NEEGS, respectively, and studied through MTT assay. Value represents mean $\pm$ SE ($n = 6$). # denotes significant differences ($P < 0.001$) between control (ethanol) versus EEGS and control (blank nanoparticles) versus NEEGS. (b) Viability of A375 cells treated with EEGS and NEEGS. Value represents mean $\pm$ SE ($n = 6$). Significant differences between EEGS and NEEGS denoted by $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*

Apoptosis assay by FACS using AnnexinV-FITC and propidium iodide staining

Data on Annexin V-FITC staining on A375 cells were in agreement with that of TUNEL assay using FACS, in respect of both EEGS and NEEGS (Figures 8a–c). NEEGS (100–200 μg)-treated cells (Figure 8c) showed noticeable apoptotic activity *vis-á-vis* EEGS (100–200 μg)-treated cells. Thus the data of both TUNEL assay and Annexin V methods complemented each other and reaffirmed the greater apoptotic potential ($P < 0.01$ and $P < 0.001$) of NEEGS than their EEGS, which also showed considerable apoptotic activities at the higher dose.

DNA ladder assay

To investigate DNA damage, genomic DNA of A375 cells treated with 100 μg and 200 μg of EEGS and NEEGS, respectively, were extracted and DNA ladders analyzed (Figure 9). As compared with the control (blank nanoparticles), the fragmentation in both EEGS and NEEGS groups (Figure 9) appeared to be more smeared, indicating more fragmentation of DNA suggestive of a greater degree

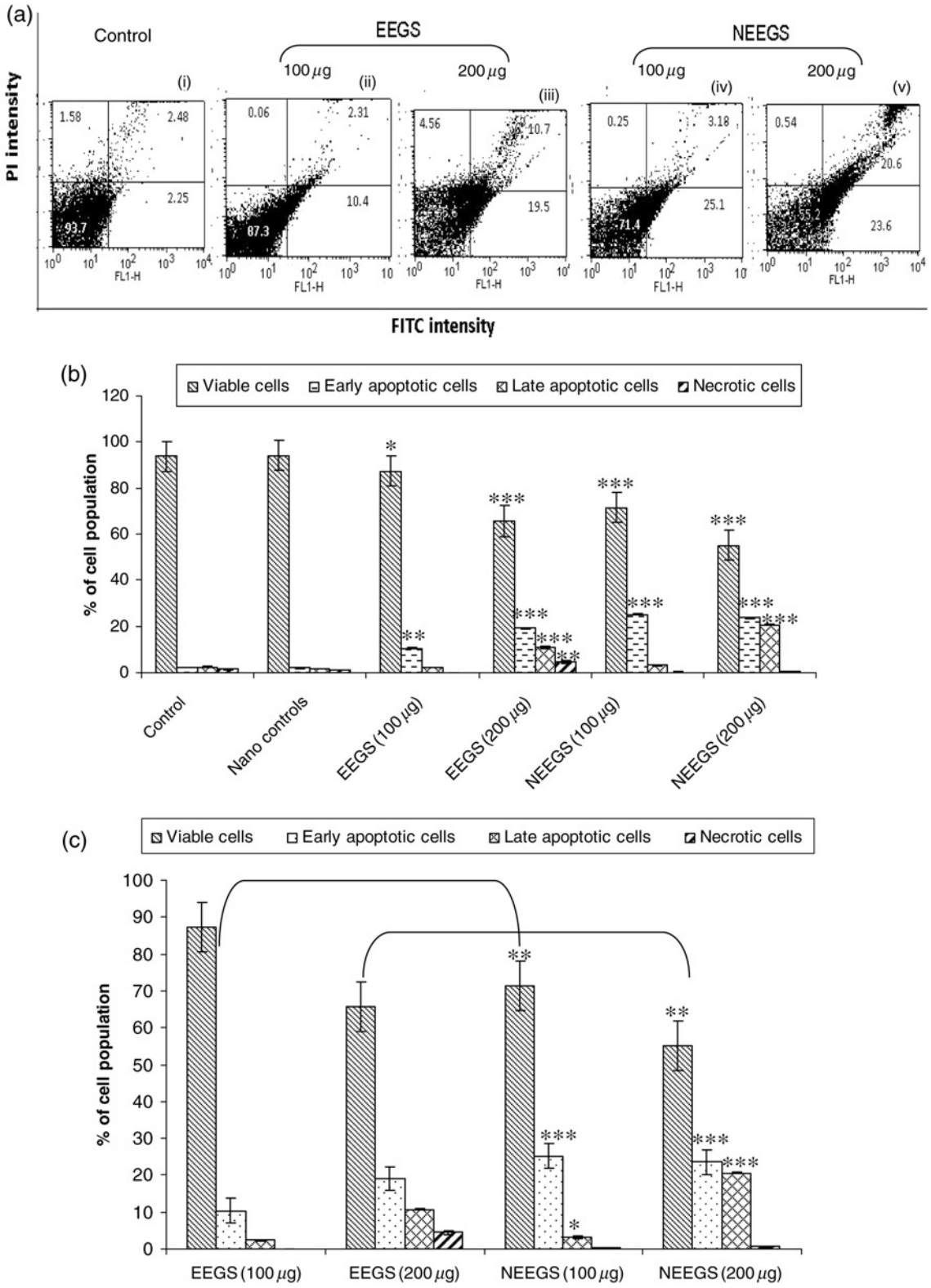

Figure 6 (a) TUNEL assay for EEGS and NEEGS-induced apoptosis in A375 cells. (i) Control cells cultured for 24 h. (ii, iii) Cells treated with two doses of EEGS (100 and 200 μg) and (iv, v) Cells treated with two doses of NEEGS (100 and 200 μg). The viable cells located in the lower left corner shows negative for both BrdU-FITC and PI. Early apoptotic cells and late apoptotic cells are in the lower right corner and upper right corner (double positive), respectively. Necrotic cells lacking a cell membrane structure are in the upper left corner (PI positive). (b) Histogram represents percentages of cell population in different phages of TUNEL assay. Value represents mean $\pm$ SE ($n = 6$). Significant differences from vehicle control (blank nanoparticles for NEEGS and ethanol for EEGS) are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (c) Quantitative analysis of TUNEL assay. Histogram represents percentages of cell population in different phages. Values represents mean $\pm$ SE ($n = 6$). Significant differences between EEGS and NEEGS are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*; TUNEL, terminal transferase dUTP nick end labeling; PI, propidium iodide

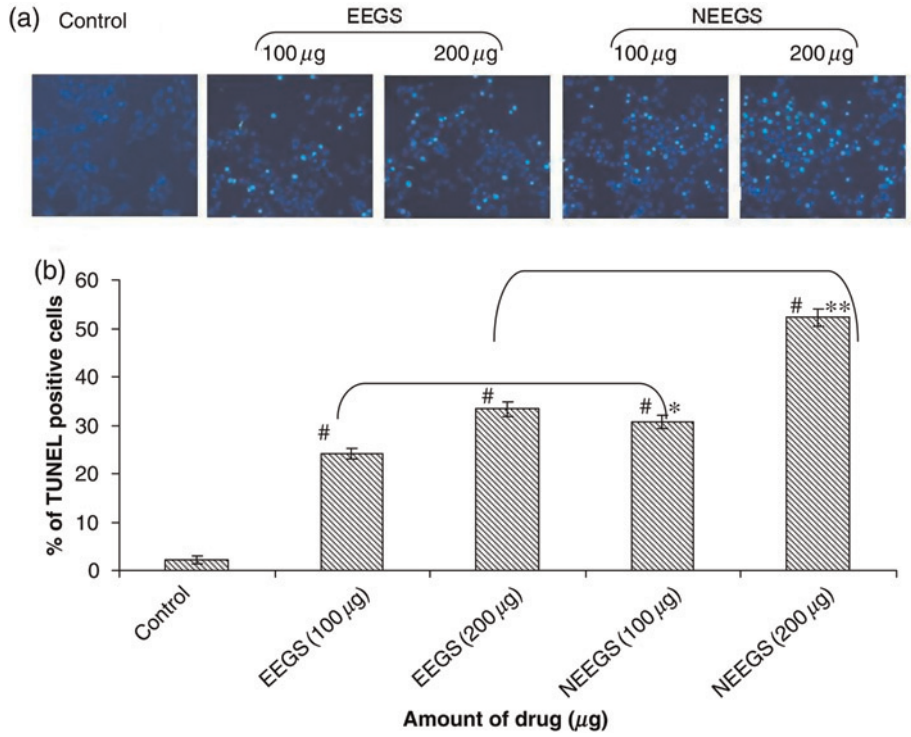

Figure 7 (a) Induction of apoptosis by EEGS and NEEGS. TUNEL-positive cells with faint green fluorescence were monitored after incubation for 24 h. (b) Histogram represents the TUNEL-positive cells. Significant differences between EEGS and NEEGS are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$. # indicates significance level ($P < 0.001$) between control versus EEGS and control versus NEEGS. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*; TUNEL, terminal transferase dUTP nick end labeling (A color version of this figure is available in the online journal)

of apoptosis. NEEGS-treated cells showed more smearing of DNA than that of EEGS-treated cells.

RT-PCR data of some key signal proteins

RT-PCR data on m-RNA levels of cyclin-D1, caspase-3, Survivin, PCNA and p53 in response to EEGS and NEEGS treatments were evaluated (Figures 10a–c). For normalization of data, a house-keeping gene for β-actin has also been considered. Cyclin-D1 is a key cell cycle regulatory protein, which governs cell cycle progression from G1 to S phase. Over-expression of cyclin-D1 is correlated with the early onset of cancer and risk of tumor progression and metastasis. Survivin, a member of the IAP family, is a bi-functional protein that suppresses apoptosis and regulates cell division. PCNA is usually regarded as a proliferation marker. The expressions of cyclin-D1, survivin and PCNA have been found to be down-regulated in all the cases, which implied antiproliferative action of the drug. p53 plays an important role in cell cycle arrest and in apoptosis. Caspases, the cytoplasmic aspartate-specific cysteine proteases, have been shown to play a central role in the apoptotic signaling pathway. Caspase-3, a member of the caspase family, was shown to play an essential role in apoptosis induced by a variety of stimuli. Finally, we examined whether p53 and caspase-3 activities were increased during NEEGS-induced apoptosis in A375 cells. The expression of p53 and caspase-3, that favors apoptosis, was up-regulated in both EEGS and NEEGS groups, the NEEGS showing greater expression. When the data between EEGS and NEEGS were further critically analyzed, the latter showed stronger effect ($P < 0.05$ and $P < 0.01$) in respect of apoptosis induction.

Discussion

The use of some TMs including homeopathy is not freely acceptable by many, mainly because of lack of experimental validation and lack of understanding of its precise mechanism of action. However, the search for safe, affordable and efficient natural plant products for chronic and difficult-to-cure diseases such as cancer has initiated a renewed interest in the world of pharmacopoeia for suitable medicinal plants. EEGS, which is used as homeopathic mother tincture, contains some 45 alkaloids categorized into some five structurally different groups.[11,12] Most of these alkaloids are poorly dissolved in aqueous solvents and toxic in nature, and should be used only in small doses. Since in homeopathy practice, small doses are always recommended for use, we wanted to examine if the efficacy of the drug could be enhanced by nanoparticle encapsulation. This would be of further benefit because the encapsulated drug could be directly carried into the cells, making it more bioactive.

Our earlier study[4] demonstrated anticancer potentials of EEGS in human HeLa cells *in vitro*. Further, the role of some nanoparticles was also predicted in making subtle changes in molecular orientation of the drug molecules

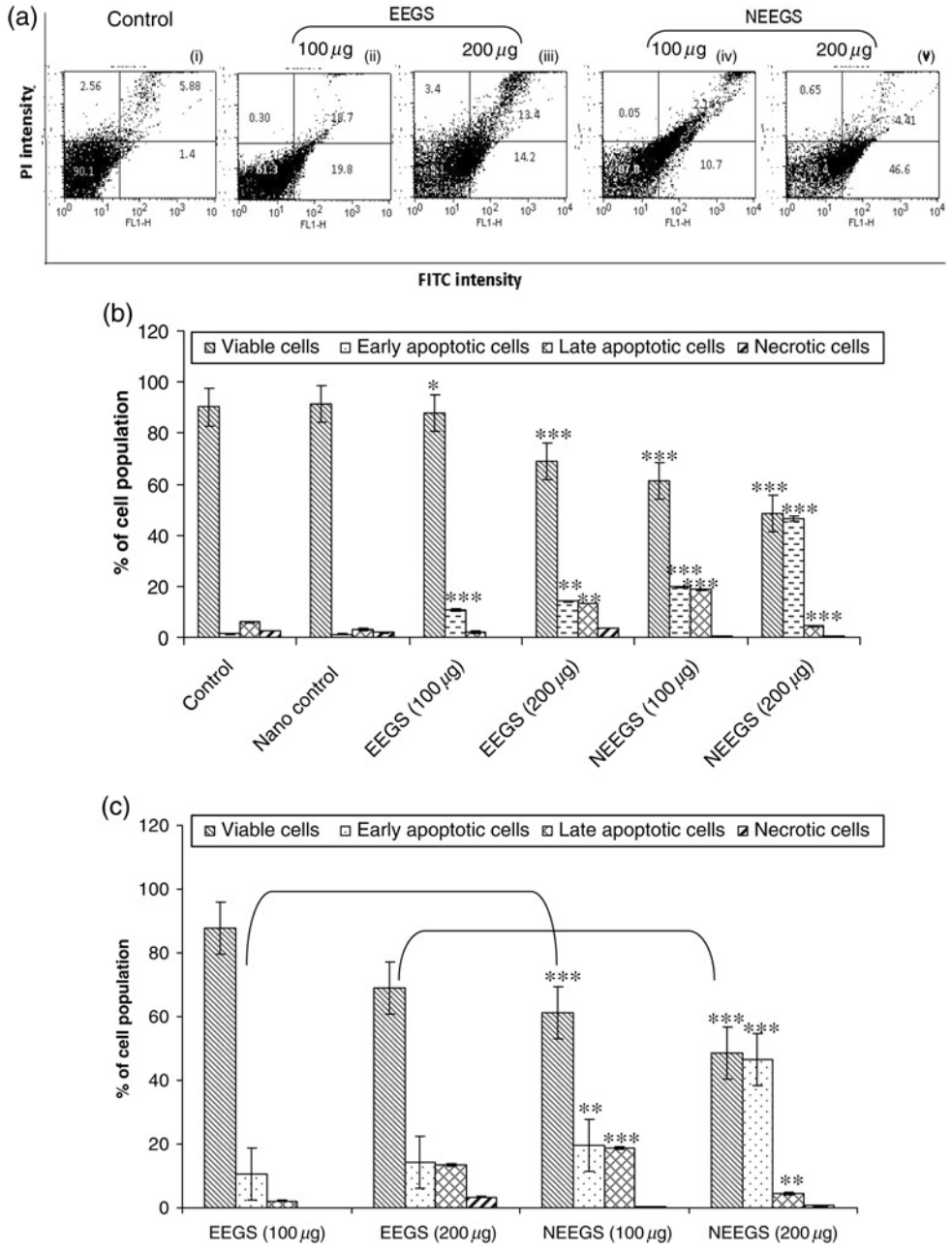

Figure 8 (a) EEGS and NEEGS-induced apoptosis in A375 cells. (i) Control cells cultured for 24 h. (ii, iii) Cells treated with two doses of EEGS (100 and 200 μg). (iv, v) Cells treated with two doses of NEEGS (100 and 200 μg). The viable cells are located in the lower left corner (negative for both annexin V-FITC and PI). (b) Histogram represents percentage of cell population in different phages by Annexin V-FITC assay. Values represent mean $\pm$ SE ($n = 6$). Significant differences from vehicle control (blank nanoparticles for NEEGS and ethanol for EEGS) are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (c) Histogram represents percentage of cell population in different phages by Annexin V-FITC assay. Values represent mean $\pm$ SE ($n = 6$). Significant differences between EEGS and NEEGS are indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*; PI, propidium iodide

and thereby biological activities. Therefore, the results of the present study assume further significance. In the present study, we confirm the anticancer potentials of EEGS in two non-toxic doses, the higher having slightly greater anticancer potentials. The results suggested that the higher dose was more efficient in inducing apoptosis as compared with the lower dose of EEGS. However, NEEGS was found to be even more potent and active as compared with EEGS. It is interesting to note that the cellular uptake of NEEGS was relatively greater and rapid (30 min) than that of EEGS (45 min). Therefore in our present formulation using a biodegradable nanoparticle encapsulation of EEGS with PLGA, we were able to increase the bioavailability and thereby improve its overall effects on cell proliferation. To our knowledge, this is the first attempt to encapsulate a mother tincture of homeopathic drug of plant origin that is known to have anticancer potentials, and the results of exposure to skin cancer A375 cells *in vitro* are encouraging for their possible therapeutic use as an anticancer agent. Incidentally, PLGA has earlier been used to encapsulate

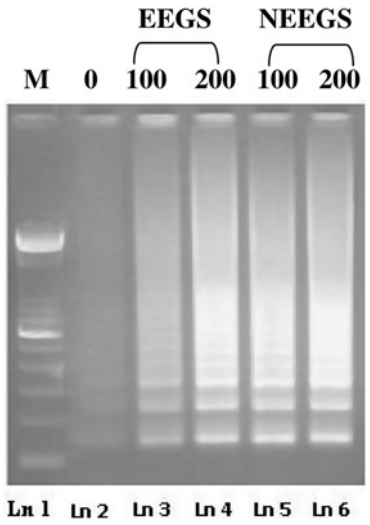

Figure 9 Genomic DNA was extracted and separated on 1% agarose gels and stained with ethidium bromide. Ln 1, control; Ln 2, EEGS (100 μg); Ln 3, EEGS (200 μg); Ln 4, NEEGS (100 μg); Ln 5, NEEGS (200 μg). EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*

other orthodox drugs, including co-enzyme Q[13] taxol[14] and camptothecin.[15] However, in the plant kingdom, nanoparticle-encapsulated curcumin, the yellow pigment present in turmeric (*Curcumin longma*), has also been reported to have enhanced cellular uptake and increased bioavailability.[16,17] In the present study we found that NEEGS was more active than the un-encapsulated mother tincture in the expression of p53 and caspase-3, two key signal proteins, and there was down-regulation of survivin, cyclin-D1 and PCNA, which would probably indicate the mechanism and pathways involved through which these drugs worked.

Further, results of Annexin V-FITC and the TUNEL assay using FACS also confirmed that the encapsulated form induced greater amount of apoptosis with less necrotic potentials than their un-encapsulated counterpart. Further studies will be needed to know if the nanoparticle-encapsulated EEGS can also be equally effective in animal models *in vivo*. Further investigation will also be necessary to test if the efficacy of the principal biologically active compound(s) can also be enhanced by PLGA nanoparticle encapsulation, by conducting experiments in both *in vivo*

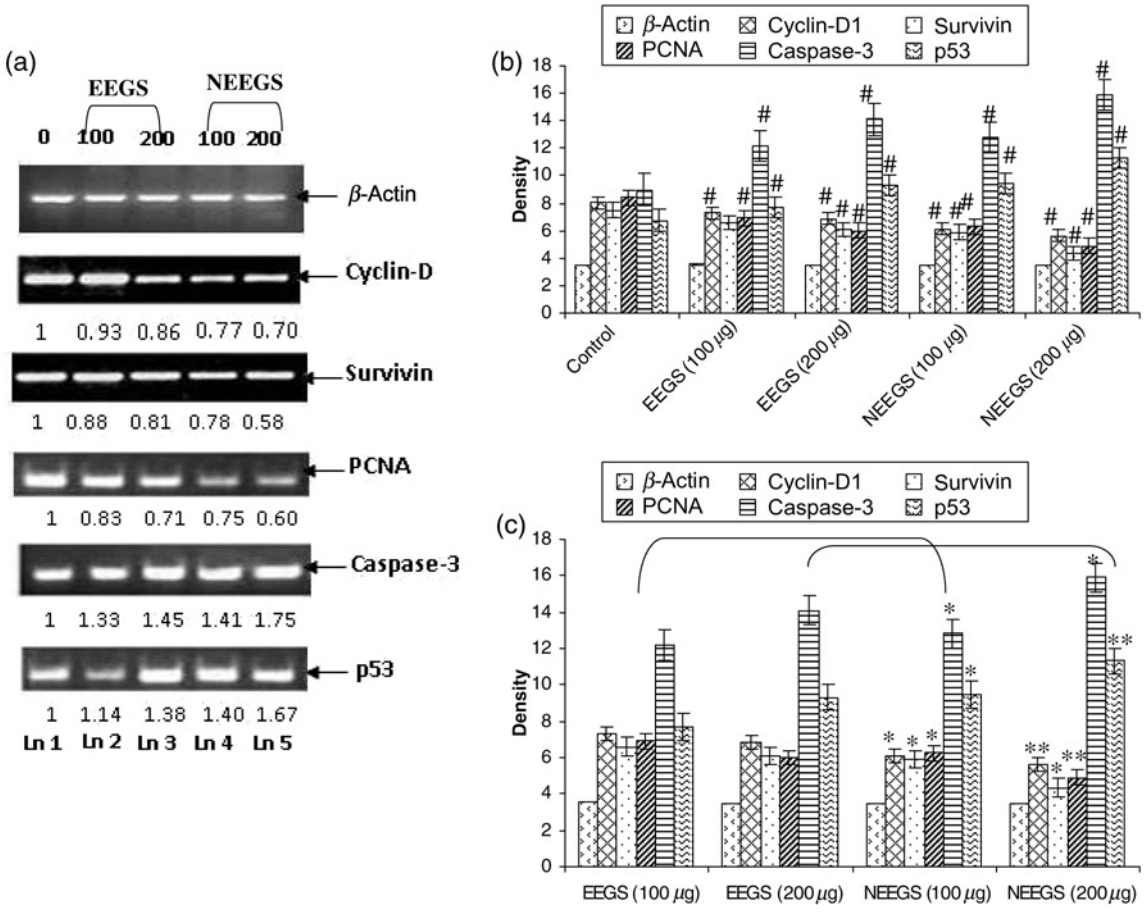

Figure 10 (a) A375 cells were incubated with EEGS (100 and 200 μg) and NEEGS (100 and 200 μg). The cells were harvested and the expression of cyclin-D1, Survivin, PCNA, caspase-3 and p53 analyzed by RT-PCR. β-Actin was used as a loading control. Ln 1, control; Ln 2, EEGS (100 μg); Ln 3, EEGS (200 μg); Ln 4, NEEGS (100 μg); Ln 5, NEEGS (200 μg). Band densities are expressed as mean $\pm$ SE of six independent experiments. (b) Histogram represents densitometric data of different mRNA expression in all groups. # represents highly significant differences ($P < 0.001$) from controls versus all groups. (c) Histogram represents densitometric data of different mRNA expression in EEGS and NEEGS-treated groups. * and ** denote significant differences ($P < 0.05$ and $P < 0.01$, respectively) between EEGS and NEEGS for both doses. EEGS, ethanolic extract of *Gelsemium sempervirens*; NEEGS, nanoparticle-encapsulated ethanolic extract of *Gelsemium sempervirens*; RT-PCR, reverse transcriptase-polymerase chain reaction

and *in vitro* systems. Works in this field are rapidly gaining ground and should prove rewarding in the areas of toxicology and therapeutics and also in drug development for dreadful diseases like cancer.

Author contributions: SSB worked on nano-encapsulation of EEGS and studied DLS, scanning electron microscopy, FACS and RT-PCR.

SP worked on the *in vitro* effects of nano-encapsulated and un-encapsulated *G. sempervirens* involving various techniques for apoptosis, cell viability, etc.

ARK-B designed the whole experiment, supervised implementation, checked results and wrote the manuscript.

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