Journal of Bioequivalence & Bioavailability
ISSN: 0975-0851

Sharma et al., J Bioequiv Availab 2016, 8:5
DOI: 10.4172/jbb.1000300

Research Article Open Access

Novel Encapsulation of Lycopene in Niosomes and Assessment of its Anti-cancer Activity

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Abstract

The lycopene is a naturally occurring bioactive constituent of *Lycopersicum esculentum* and its use in the prevention of cancer and diabetes. Developing effective therapy using a natural product like lycopene against cancer is an active area of research. The pure lycopene is susceptible to light, heat and oxidants, which limit its therapeutic applications. In the present investigation, a novel approach for lycopene encapsulation using glass wool (adsorption-hydration method) is represented. The niosome formulation was prepared to preserve the activity of lycopene and to improve the bioavailability. The niosomes were characterized by *in vitro* studies like entrapment efficiency, particle size, drug release profile, zeta potential, stability studies, etc., and *in vivo* bioavailability study. The anti-proliferative efficacy of formulation was evaluated against MCF-7 and HeLa cell lines which exhibited an excellent response in a dose-dependent manner. The apoptosis assay demonstrated that anti-proliferative activity occurred due to an apoptotic pathway, which revealed the potential in formulation development of lycopene against cancer. The overall results indicate that the methodology for the formulation of lycopene niosomes is promising and very effective. It has the potential for wider applications along with the fact that, the method used for active drug loading into niosomes strongly influences the pharmaceutical performance of the formulation.

Keywords: Lycopene formulation; Oxidants; Niosomes; Nanoparticles; Anticancer activity

Introduction

Lycopene is a major carotenoid pigment present in ripe tomatoes (*Lycopersicum esculentum*) having characteristic red color and has been the focus of considerable attention for its potential health benefits [1,2]. The lycopene is a polyunsaturated hydrocarbon (an unsubstituted alkene), and structurally it is a tetra-terpene assembled from eight isoprene units. It has eleven conjugated double bonds give it its deep red color and is responsible for its antioxidant activity. It reduces oxidative DNA damage and results in the lower prostate-specific antigen, which prevents the cancer. Results from epidemiological and experimental studies support the view that lycopene may provide protection against cardiovascular disease and cancer [3-5]. However, the presence of unsaturated bonds in the molecular structure of lycopene, make it susceptible to oxidants, light, and heat, which can be easily deteriorated when exposed to such factors [6-8]. Therefore, the free lycopene must be protected from chemical damage before its application. To overcome such problems various drug delivery systems like liposome, niosome, etc., have been found promising for better and effective with various degree of success.

Liposomes have shown advantage as drug carriers, but they are associated with problems related to physical stability such as fusion, aggregation, sedimentation and leakage on storage [9]. The noisome potentially improve the bioavailability, which can enhance the rate and extent of solubilization into aqueous intestinal fluids Like phospholipids; the nonionic surfactants are able to form vesicular systems (niosomes) when dispersed in aqueous media [10,11]. Niosomal vesicle has potential to encapsulate both lipophilic and hydrophilic drugs and protect them *in vivo* [12]. In many reported studies, it is shown that niosomes behave *in vivo* like liposomes as they prolong the circulation of encapsulated drug, altering its organ distribution and metabolic stability [13]. Niosomes have better pharmacokinetic and pharmacodynamic profile due to the higher affinity of the cholesterol to the skin phospholipid, this not only improves the absorption of the active compound but also increases the duration of the activity as the complex slowly releases the active principle. They offer several advantages over liposomes such as higher chemical stability, intrinsic skin penetration enhancing properties and lower cost [14]. The ease of transfer and good drug carrying potential make niosomes a versatile and suitable delivery system for lycopene [15].

In the present study, we have formulated the lycopene Nano-noisome by developing a novel adsorption–hydration method. This method is an attempt to preserve the lycopene activity in niosomes, using glass wool as a platform for interaction between lycopene and wall material which will circumvent its formulation difficulties. The protective mechanism therein is to form a membrane (wall system) to enclose droplets of the adsorbed lycopene (core). For wall system, span 60 and cholesterol are used, which acts as membrane stabilizer to assist solute retention by forming a bilayer, and improves the adhesion force between the wall and core materials. Hence, in this study, the nano-niosome particles for lycopene was prepared with a novel approach and its actual capabilities were studied by analyzing entrapment efficiency, release profile, stability, zeta potential, and bio-availability. *In vitro* anti-cancer activity of lycopene-loaded nano-niosomes was evaluated for its biological efficiency.

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Received August 03, 2016; **Accepted** August 03, 2016; **Published** August 20, 2016

Citation: Sharma PK, Saxena P, Jaswanth A, Chalamaiah M, Tekade KR, et al. (2016) Novel Encapsulation of Lycopene in Niosomes and Assessment of its Anti-cancer Activity. J Bioequiv Availab 8: 224-232. doi: 10.4172/jbb.1000300

Material and Methods

Material

Lycopene extract was isolated from *Lycopersicum esculentum* (Bhopal, India). Span 60, cholesterol, chloroform, n-hexane, ethanol, acetone, were obtained from Merck Limited (Mumbai, India). Cisplatin was purchased from Sigma-Aldrich (India). All other chemicals were of analytical grade. All solvents used were of HPLC grade.

Isolation of lycopene

The five kilogram of fresh ripe tomatoes *(L. esculentum)* was collected from a local market in Bhopal, India during the month of November 2011 and was authenticated by Department of Botany, Safia Science College Bhopal, India. The voucher specimen (568/BOI/SAFIA/12) was deposited department of pharmaceutics, Technocrats Institute of Technology-Pharmacy Bhopal, India. The ripe tomatoes were stored at 4°C and were used within 48 h for isolation of lycopene. The peels of tomato were separated using blanching method, it involves immersing of tomatoes in boiling water for 1-2 min, followed by rapid cooling and hand peeling. The removed peels were dried at room temperature (24-25°C) for 2-3 h and then packed in zip-lock polyethylene bags. The dried peels were stored at 4°C before grinding with a small amount of solvent system (n-hexane: acetone: ethanol in 2:1:1 v/v) in the presence of CO_2. It was then sonicated using ultrasonic crusher followed by magnetic stirring for 4 h in an inert environment (CO_2) under dark conditions. The resulting extract was collected in an amber color glass container with the presence of CO_2 environment and stored at 4°Cfor further processing [16-18]. The extract was then dried using rotary flash evaporator by heating at 60°C at 50 rpm in dark condition.

Preparation of lycopene nano-niosomes

The lycopene Nano-niosomes were prepared by developing a novel method named Adsorption-Hydration technique. In this technique, the pure extract was collected and dispersed in a small amount of solvent system (n-hexane: acetone: ethanol in 2:1:1 v/v). About 22 mL of span 60 and cholesterol (1:1 mol) were added to10 mL of above solvent system as organic phase having wall material. This phase was absorbed over inert media (3 g glass wool) with constant stirring at 60°C. The 10 mL aqueous phase (mannitol solution: pH-7.4) was then added slowly to the lycopene adsorbed glass wool with vigorous shaking at 60°C temperature followed by sonication using an ultrasonic cell crusher (classic, sonicator, Lark Innovative Fine Teknowledge, Chennai, India) at 20 W output for 60 sec/cycle in three cycles to protect the solution from heat buildup. Then it was kept for 2-3 h for niosomes to formulate. The suspension of self-assembled, lycopene-loaded nano-niosomes was then filtered using 0.45-mm, Whatman filter (No-1) to remove glass wool. The filtered suspension was then processed through column chromatography (Sephadex G-50 column) to separate un-entrapped lycopene. Finally, the formulation was collected, lyophilized (Lyodel, Delvac, India) and stored in an air tight high-density plastic container.

Characterization

The pure lycopene extract was characterized for its physical appearance, melting point, solubility and absorption maxima (λ_{max}). The lycopene-loaded nano-niosomes were characterized in terms of particle size, entrapment efficiency, zeta potential, *in vitro* drug release and *in vivo* bioavailability studies. Its biological activity was characterized by anti-cancer study on cell lines.

Characterization of lycopene

Physical appearance and microscopic characteristics: The isolated pure lycopene extract was observed morphologically and examined under Clinical Microscope (Olympus) for its appearance and microscopic characteristics.

Melting point: A small quantity of lycopene powder was inserted into a fusion tube. This tube was placed in melting point apparatus. The temperature of the apparatus was gradually increased such that, it reaches the temperature at which powder starts melting and the range was observed.

Solubility: The solubility study was performed by shaking 10 mg lycopene in 10 mL of different solvents like water, methanol, chloroform, n-hexane, and ethanol and petroleum ether, in a conical flask with the help of mechanical shaker for 30 mins and was observed for its solubility.

Determination of absorption maxima (λ_{max}): Ten mg of lycopene were dissolved in 10 mL of n-hexane in a volumetric flask. Then, 1 mL of this stock solution was pipetted out in 100 mL volumetric flask and volume was made up to 100 mL mark with n-hexane. The resulting solution was scanned between 400 to 600 nm using UV spectrophotometer (Shimadzu 1700) and the absorption spectrum (λ_{max}) was obtained. The standard curve of the lycopene in n-hexane was prepared at the observed absorption maxima. The regressed calibration curve was plotted and observed for linearity and along with concentration range for beer lamberts law.

Characterization of lycopene-loaded niosomes

Particle size analysis and zeta potential determination: A drop of lycopene niosome suspension was spray dried on a clean glass slide (1 × 1 cm) and placed in a circular metallic sample holder. The slide was fixed with colloidal silver and Cu tape is used to mount sample to the stub. After that, it was coated with gold under vacuum condition and observed using JSM-6390 Scanning Electron Microscope (JEOL, USA) for evaluation of their particle size, outer surface and particle size distribution. Zeta potential of vesicles was determined by using Zetasizer (Malvern Instruments, UK). Niosomal suspensions were properly diluted with distilled water and dropped into the Zetasizer electrophoretic cell. Each sample was measured six times at 25 ± 0.1°C.

Entrapment efficiency (EE): The lycopene nano-noisome suspension (0.5 mL) was loaded onto a Sephadex G50 column (granulometry 20-80 μm) and was eluted with distilled water. The lycopene-loaded noisome were eluted as a red-orange liquid, while free lycopene remained bound to the column. Vesicles were disrupted through the addition of 0.05 mL TritonX-100 and ultra-centrifugation at 120000 g for 1 h (Beckman L8-M, Beckman Coulter s.r.l., Milan Italy). The total lycopene in niosomal dispersion and the lycopene entrapped in noisome were recovered and assayed by UV spectrophotometric method. The encapsulation efficiency was calculated using the following equation:

$$EE\% = \frac{[\text{Encapsulated drug present after chromatography}]}{[\text{Total drug present before chromatography}]} \times 100$$

Stability studies of lycopene nano-niosomes: Stability study of lycopene-loaded nano-niosome particles and the unloaded nano-niosomes was carried out at 4°C and 25°C, and at the different pH environment. Particle size, zeta potential and encapsulation efficiency (determined after separation of loaded from the unloaded drug by

size exclusion chromatography) were measured immediately after the preparation and on every 15th day during the three-month time period.

***In vitro* drug release profile:** The *in vitro* release of lycopene from niosomes was carried out using dialysis system consisting of a dialysis bag and a receptor compartment. The overnight hydrated dialysis membrane was fixed to the funnel of diffusion cell assembly and the setup was configured. The niosomal suspension (3 mL) was taken in donor compartment (dialysis bag) closed by dialysis bag closure clip and placed in a beaker (receptor compartment) having 250 mL phosphate buffer saline (PBS) (pH 7.4). The beaker was stirred at 50 rpm (at 37 ± 1°C). During sampling, 4 mL sample was withdrawn periodically at 0, 1, 2, 3, 5, 7, 8, 10, 24, 36, 48, 60, 72 h and replaced by equal volume of fresh PBS. The withdrawn samples were diluted and analyzed for drug content using UV spectrophotometer at λ_{max} (205 nm). Phosphate buffer saline was used as blank. The lycopene release pattern was observed for 72 h [19-21].

Bioavailability study of lycopene-loaded nano-niosomes

Animals: Wistar rats (150 g) of either sex, approximately 6-8 weeks old, were procured from Institute of Animal Health and Veterinary Biologicals, Mhow (Indore, India). The animals were maintained in polypropylene cages with 12 h light and dark cycles at a temperature of 23 ± 2°C and a humidity of 55 ± 5%. They were maintained on laboratory diet and water *ad libitum*. The animals were acclimatized to laboratory condition for one week before starting the experiment. The experiment was carried out in accordance with a protocol approved by Institutional Animal Ethics Committee (TIT/IAEC/831/PHARMACEUTICS/2012/07) at TIT-Pharmacy, Bhopal, India.

Experimental design: The three male rats were randomly taken into two groups (n=3) (Lycored marketed formulation and nano-niosomes formulation) and fasted for 12 h with free access to ad libitum water before administration. They were given a dose of 100 mg/kg via the oral route (Lycored and nano-niosomes formulation, respectively). The blood samples were collected from the retro-orbital plexus at predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h post dosing) into heparinized tubes. Plasma was separated from the whole blood by centrifugation at 10,000 rpm for 10 min and the resultant supernatant kept at −80°C for further analysis. The results of the pharmacokinetic study were analyzed by RP-HPLC.

Analysis of plasma: The plasma concentration of Lycopene was determined by a validated HPLC system equipped. Plasma (100 µL) was extracted with 100 µL ethanol and 200 µL hexane and samples were vortexed for 2 min and centrifuged for 5 min at 3000 rpm and 4°C. The organic phase was separated and dried in the absence of light under an atmosphere of nitrogen. Samples were reconstituted with mobile phase. The amounts of lycopene were measured by HPLC using a Shimadzu HPLC (Kyoto, Japan) equipped with two LC-10AD pumps, a rheodyne injector with photodiode array detector. The following conditions were employed for HPLC: A reverse phase C-18 column (phenomenax, luna 250 × 4.6 mm, 5 µm) eluted with an isocratic solvent system consisting of acetonitrile:THF:methanol (68:22: 10, v/v/v), with 0.025% (w/v) ammonium acetate at 1 mL/min. Lycopene was detected at 445 nm. The detection limit of the assay was 5 pmol/injection, based on a signal-to-noise ratio of 3:1. The quantification limit of the assay was determined as the concentration equal to six times the value of the signal-to-noise ratio and was 8 pmol/injection.

Pharmacokinetics: The pharmacokinetic parameters were calculated using non-compartmental analysis (WinNonlin 4.0, Pharsight, Mountain View, CA). The area under the plasma concentration-time curve from time 0 to time infinity ($AUC_{0\to24}$) was calculated using the linear/log trapezoidal rule-extrapolation method, in which the linear trapezoidal rule was used for the calculation of the area during the ascending phase with the logarithmic trapezoidal rule for the declining phase. The peak plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration vs. time profiles. Relative bioavailability (%) was calculated as the ratio of $AUC_{0\to24}$ obtained after oral administration of lycopene Nano-noisome and Lycored, respectively.

In vitro evaluation of the anticancer activity

Cell culture: MCF-7 and HeLa (Human breast cancer cell line, NCCS Pune) cells were maintained in medium (RPMI 1640) supplemented with 10% (v/v)heat-inactivated fetal calf serum (FCS) (PAN Biotech GmbH, Aidenbach, Germany), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen, France) and 2 mML-Glutamine (Invitrogen, France) in a controlled atmosphere of 5% CO_2, and humidified air at 37°C.

MTT cytotoxicity assay: The *in vitro* cellular effect of synthesized nano-niosomes loaded with lycopene using 3-(4,5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was investigated on MCF-7 and HeLacell lines. Briefly, 5×10^3 cells/well were seeded in 96-well plates in 200 µL Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and incubated for 24 h at 37°C in CO_2 incubator. Lycopene loaded niosomes were diluted to various concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM) in culture medium, and added to the wells. After 72 h of incubation, 10 µL MTT (5 mg/mL) was added to each well and the plates were further incubated for 2 h after, followed by addition of 100 µL of lysing-buffer, and the plate was again incubated for a period of 2 h. Then, the supernatant from each well was carefully removed, formazan crystals were dissolved in 100 µL of DMSO and absorbance at 570 nm was recorded (Molecular Devices, USA). Percentage cell viability was calculated using following formula.

$$\%\,\text{Cell Viability} = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Cell topographic study: The alternation in cell topography was determined by *in vitro* incubation of HeLa cells with lycopene-loaded niosomes. Niosomes were incubated with HeLa cell lines for 24 h, the cultured cells were utilized for assessing the influence of formulation on the structure as well as cellular colonies. Changes were observed using a confocal microscope (Leica, Germany).

Statistical analysis: All the statistical analysis was performed with GraphPad Software (Version 3.0, Graph Pad Software, California, USA) using one-way analysis of variance(ANOVA) followed by Dunnett's't' test for multiple comparisons and student's t-test for a single comparison. The pharmacokinetic data was calculated using WinNonlin (Version 4.0, Pharsight, Mountain View, CA). Significance was expressed as $^{*}p<0.01$; $^{**}p<0.001$. The results are expressed as Mean ± Standard error (S.E).

Results

The extraction yield of pure lycopene from tomato peel was found to be 571 µg/mL of the solvent system.

Isolation and characterization of lycopene

The isolated pure lycopene extract appeared as brick red color crystals and found odorless in nature as given in Table 1. The microscopic evaluation revealed the structure of lycopene as long deep

red needles, as shown in Figure 1. The melting point range was found to be 172-173°C which complies with Indian Pharmacopoeia (IP) 2007. The solubility of lycopene was found to be more in organic solvents as given in Table 2; the solubility was studied with various solvents and it complies with literature. The purity of lycopene was determined with UV spectrophotometric method using absorption spectra (λ_{max}); the λ_{max} was obtained at 504 nm (Figure 2) which is comparable with the reference spectrum of the lycopene in IP 2007. The regressed calibration curve showed good linearity and follows beer lamberts law in a concentration range from 2 to 10 µg/mL. The initial characterization results confirm the presence of pure lycopene in the extract.

Characterization of lycopene-loaded niosomes

The pure lycopene extract isolated from tomatoes peel was adsorbed in glass wool (Figure 3) (adsorption–hydration method) and then lycopene nano-niosome formulation was developed and characterized.

Particle size, size distribution and zeta potential: As shown in Figures 4a-4c, the particle size and size distribution of lycopene nano-niosomes were ranged from 170 to 230 nm with average particle size 200 ± 40 nm. Morphology of lycopene niosomes was studied using scanning electron microscopy (SEM). The particles observed were spherical and morphologically similar. In order to study the modality of both lycopene-loaded and unloaded niosomal nanoparticles, the zeta potential value was studied under different pH conditions and the changes were not significantly different, indicating the absence of ionization phenomena of the membrane components (Table 3).

Entrapment efficiency (EE): The nano-noisome formulation of lycopene extract was evaluated for entrapment efficiency. The entrapment efficiency of the reconstituted niosomes was detected with UV spectrophotometric method and the mean value of the EE was found to be 62.76 ± 2% (n=3). This might be due to more sites for interaction between lycopene adsorbed in glass wool (Figure 3) with wall materials during formulation development.

Parameters	Observations
Physical appearance	Crystalline powder
Colour	Brick red
Odour	Odorless

Table 1: Physical appearance of lycopene.

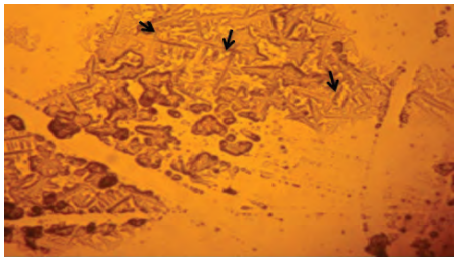

Figure 1: Figure showing microscopic crystalline structure of pure lycopene.

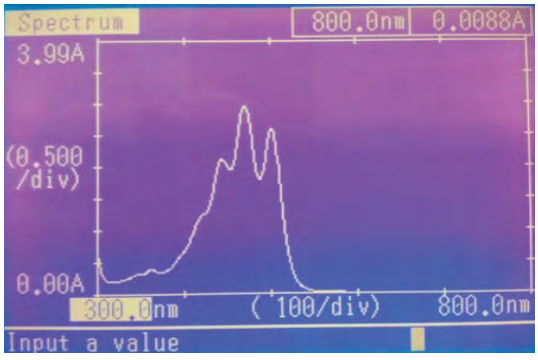

Figure 2: UV spectra of pure lycopene extract.

Figure 3: Photo of lycopene adsorbed inglasswool.

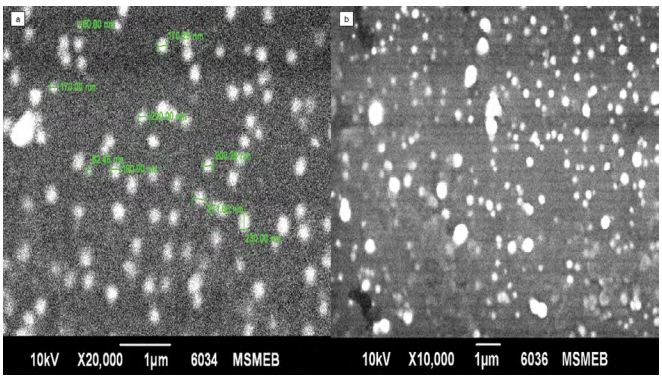

Figure 4: SEM image of lycopene niosomes, (a) Particle size of lycopene niosomes (20,000X) showing uniform distribution of particle size, (b) Over all uniform size distribution of lycopene niosomes (10,000X).

Solvent	Results
Water	Insoluble
Chloroform	Soluble
N-Hexane	Highly Soluble
Methanol	Insoluble
Ethanol	Insoluble
Petroleum ether	Highly Soluble

Table 2: Solubility profile of lycopene in various solvents.

Sample	Z pot (pH=4)	Z pot (pH=5.6)	Z pot (pH=9)
Empty niosomal nanoparticles	-1.83 ± 1.1	-2.25 ± 1.2	-2.51 ± 1.3
Lycopene niosomal nanoparticles	-2.06 ± 1.2	-2.46 ± 1.4	-2.23 ± 1.1

Table 3: Effect of pH changes on zeta potential (Z pot) of lycopenenano-niosomes (n=5).

Stability studies of lycopene nano-niosomes: The stability of both unloaded and lycopene-loaded nano-niosomes were monitored in terms of vesicle particle size and zeta potential during three-month storage time at both 4 and 25°C, as given in Table 4. Entrapment efficiency of the formulation during storage was also checked, following the separation of free drug by size exclusion chromatography. The effect of pH variations on

zeta potential of both lycopene-loaded and unloaded niosomal dispersions was studied. The zeta potential of unloaded nano-noisome particles did not change in an important way at different pH conditions ranging from 4 to 9, indicating the absence of ionization phenomena of the membrane components, as reported in Table 3.

***In vitro* drug release study:** The drug release profile of lycopene-loaded nano-niosome formulation is shown in Figure 5. It was observed that the membrane bound lycopene was released initially at a faster rate, than encapsulated lycopene from the formulation. In the first 24 h, an initial lycopene release was about 19% of total entrapped lycopene in receptor compartment from the dialysis bag. During the next 48 h, 48.3% of the total lycopene was released from the donor compartment/ dialysis bag having lycopene nano-niosomes. The mechanism of lycopene release from niosomal formulations was determined by the following mathematical models: Zero-order kinetics, First-order kinetics, Higuchi kinetics, Korsmeyer-Peppas and Hixson-Crowel models as given in Table 5 (Figure 6). The sustained release of lycopene from nano-niosomes particles of membrane bound and encapsulated lycopene is evidently attributing to the prolonged release function of the niosomes. This implies that lycopene in the present formulation has a slow and prolong release profile, which maintains an optimum concentration for maximum activity for long duration both *in vitro* and *in vivo*, which is very important for different pharmaceutical and nutraceutical applications.

Bio-availability study

For the oral delivery of poorly water-soluble chemicals, there are at least two major barriers, including the pre-epithelial, unstirred aqueous layer and poor membrane permeability. To explain the possible improvement in the pharmacokinetic behavior of nano-niosomes, time evolution of plasma lycopene profile was studied by HPLC analysis after oral administration of water-suspended lycopene nano-niosomes (100 mg/kg) in rats (Figure 7). The relevant pharmacokinetic parameters for orally administered nano-niosomes, including C_{max}, T_{max}, and AUC_{0-24} are listed in Table 6. Oral administration of the Lycored resulted in gradual elevation of plasma lycopene levels up to C_{max}19.0 µg/L, and the AUC_{0-24} value was calculated to be 341.15 µg.h/L in comparison with lycopene nano-niosomes (100 mg/kg) formulation where C_{max} 64.6 µg/L, and AUC_{0-24} value was calculated to be 1355.06 µg.h/L. The relative bioavailability of lycopene nano-niosomes in rats was calculated to be 397.19%. This observation was not surprising considering the good solubility of lycopene nano-niosomes in aqueous solutions and its absorption processes [22-24].

Sample	Size (nm (n=6)		Z Potential (n=6)		EE% (n=3)	
	Day 0	Day 90	Day 0	Day 90	Day 0	Day 90
Empty, 4ºC	80 ± 4	84 ± 6	-1.9 ± 1.3	-2.3 ± 1.2	--	--
Empty, 25ºC	82 ± 4	86 ± 7	-1.9 ± 1.3	-1.7 ± 0.9	--	--
Loaded, 4ºC	180 ± 4	183 ± 7	-2.1 ± 1.2	-2.2 ± 1.2	62.7 ± 2	60.2 ± 3
Loaded, 25ºC	220 ± 4	223 ± 8	-2.1 ± 1.2	-2.1 ± 1.2	62.7 ± 2	59.1 ± 3

Table 4: Effect on stability at 4ºC or room temperature (25ºC) on size, zeta potential and entrapment efficiency of empty and lycopene nano-niosomes.

Model Name	Regression value
Zero order release	0.9906
First order release	0.8034
Higuchi model	0.9233
Hixson-Crowell model	0.9645
Korsmeyer-Pappas model	0.9915

Table 5: Regression value of mathematical model.

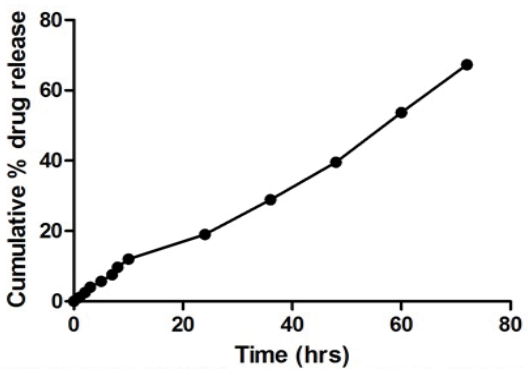

Figure 5: Graph showing *in vitro* cumulative percentage drug release profile of lycopene niosomes.

Anti-cancer activity

The evaluation of *in vitro* anticancer activity of lycopene-loaded niosomes was performed against HeLa cells and MCF cells; MTT assay and apoptosis experiment were carried out. Cell cytotoxic activity of synthesized nano-niosomes loaded with lycopene, marketed lycopene formulation (Lycored) and Cisplatin was examined by MTT assay. The percentage of viable HeLa and MCF cells incubated with niosomes and marketed formulation of lycopene (Lycored) at different concentrations for 72 h are shown in Figures 8a and 8b. In both HeLacells and MCF cells, the cell viability greatly decreased when the cells were incubated with the lycopene nano-niosomes. The minimum safe concentration (MSC) value of lycopene for MCF-7 (breast cancer cell line) and HeLa (cervical cancer cell line) cell lines were found to be 22.62 µm and 18.01 µm, respectively (Table 7). The cell topographic study was performed and cultured cells (HeLa cells) were assessed for the influence of formulation on structure, as well as cellular colonies analyzed under confocal microscope, as shown in Figure 9a-9c. As shown in Figure 9, after treatment with the marketed lycopene (Lycored) and lycopene-loaded nano-niosomes, both cells exhibited typical features of apoptosis, such as appearing of chromatin condensation and apoptotic bodies [25,26]. The numbers of apoptotic cells incubated with 100 µM concentration of lycopene-loaded nano-niosomes (Figure 9c) are compared to that treated with marketed lycopene (Lycored) (Figure 9b). On the other hand, in the control group, the cells had no morphological changes (Figure 9a).

Discussion

Lycopene is a bioactive molecule which is highly susceptible to environmental (physical and chemical) factors, which leads to loss of its potential applications. It can play an important role in human health, in particular, as a dietary antioxidant with free radical scavenging activity. Intake of lycopene reduces oxidative DNA damage and promotes health. The niosomes has the potential for better pharmacokinetic and pharmacodynamics profile than other conventional formulation of botanical extracts due to the higher affinity to the skin phospholipids. This not only improves the absorption of the active compound but also increases the duration of the activity as the complex slowly releases the active principle. The niosome formulation is more stable and releases the drug at controlled rate which makes it a good drug carrier and a versatile delivery system.

During the isolation of lycopene, we studied the influence of solvents and found that usage of n-hexane did not produce any significant difference when compared to other solvents in extraction efficiency. In

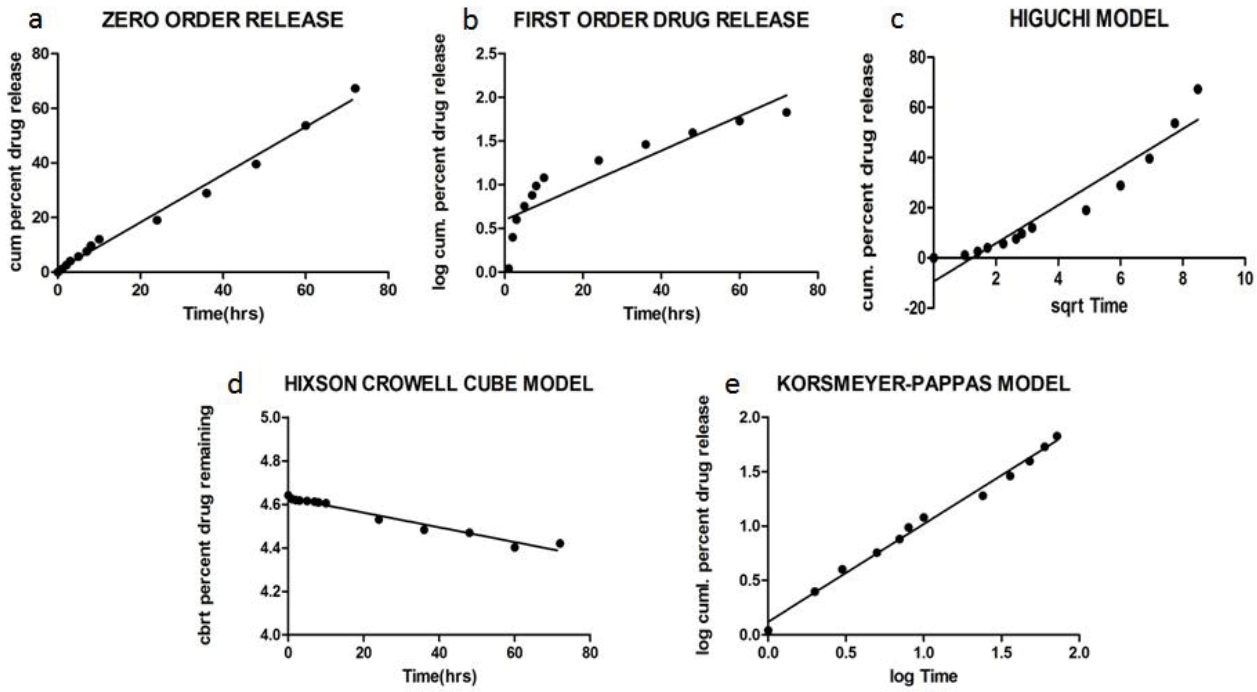

Figure 6: Mathematical modeling of drug release profile, (a) Showing zero order release profile, (b) Showing first order release profile, (c) Showing higuchi model release profile, (d) Showing hixsoncrowell cube model release profile, (e) Showing korsmeyerpappas model release profile.

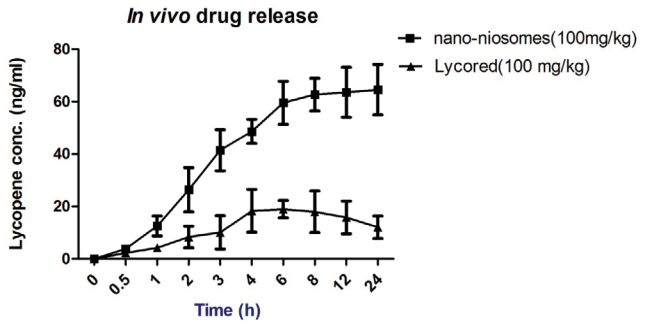

Figure 7: Graph showing *in vivo* blood plasmalevel profile of lycopene nano-niosomes.

S. No.	Parameter	Lycored®	Lycopene nano-niosomes formulation
1	C_{max} (µg/L)	19.0	64.6
2	T_{max} (h)	6	24
3	AUC_{0-24} (µg.h/L)	341.15	1355.06[a]
4	RelBA[b]	100%	397.19%

[a]Statistically different when compared to Lycored ($p<0.05$).
[b]Rel BA was the relative bioavailability (%) expressed in comparison to the Lycored$AUC_{0\rightarrow24}$ obtained after oral administration.

Table 6: Details of pharmacokinetic parameters obtained after oral administration of lycopene nano-niosomesand Lycored® to fasted rats. Lycopene was administered at a dose of 100 mg/kg (Mean).

contrast, the mixture of n-hexane:acetone:ethanol (2:1:1 v/v) appeared to be much more effective since n-hexane has a high affinity for lycopene while acetone and ethanol have high hydrogen bonding capability. The large alkalinity value causes the swelling of the plant tissue, thus

Cell Line	Formulations	MSC (µM)	IC_{50} (µM)
MCF-7	Lycopene Nano-niosome Formulation	22.62	108.13
	Marketed Lycopene Formulation (Lycored™)	55.17	178.92
	Cisplatin	3.12	43.69
HeLa	Lycopene Nano-niosome Formulation	18.01	90.03
	Marketed Lycopene Formulation (Lycored™)	52.37	140.87
	Cisplatin	2.16	39.05

Table 7: MSC and IC50 value of lycopene formulations in two cancer cell lines.

facilitating solvent penetration [16]. The main causes of lycopene degradation during processing or formulation is isomerization and oxidation, under the influence of heat, light, and oxygen, against which noisome can act as a potential system. The attempt for the development of lycopene encapsulated noisome consisted nonionic surfactant (span 60) and cholesterol. These excipients solubilized and stabilized the lycopene and provide high resistance to oxidative stress induced by different sources of free radicals [27].

The nano-noisome preparation by adsorption-hydration method was very effective in formulation of uniform size particles with high entrapment efficiency. These results would be attributed due to maximum surface area of contact achieved during preparation of wall material and lycopene. For wall system, span 60 was a good choice due to its excellent physicochemical properties including emulsification, film-formation, water-solubility, edibility and biodegradation. The higher alkyl chain length and higher phase transition temperature offered by selected excipients rendered excellent entrapment efficiency. In addition, cholesterol was used as a significant component of wall material, which can act as membrane stabilizer to assist solute retention by forming a bilayer. Thus it provides better entrapment efficiency, stability as well as improves the adhesion force between

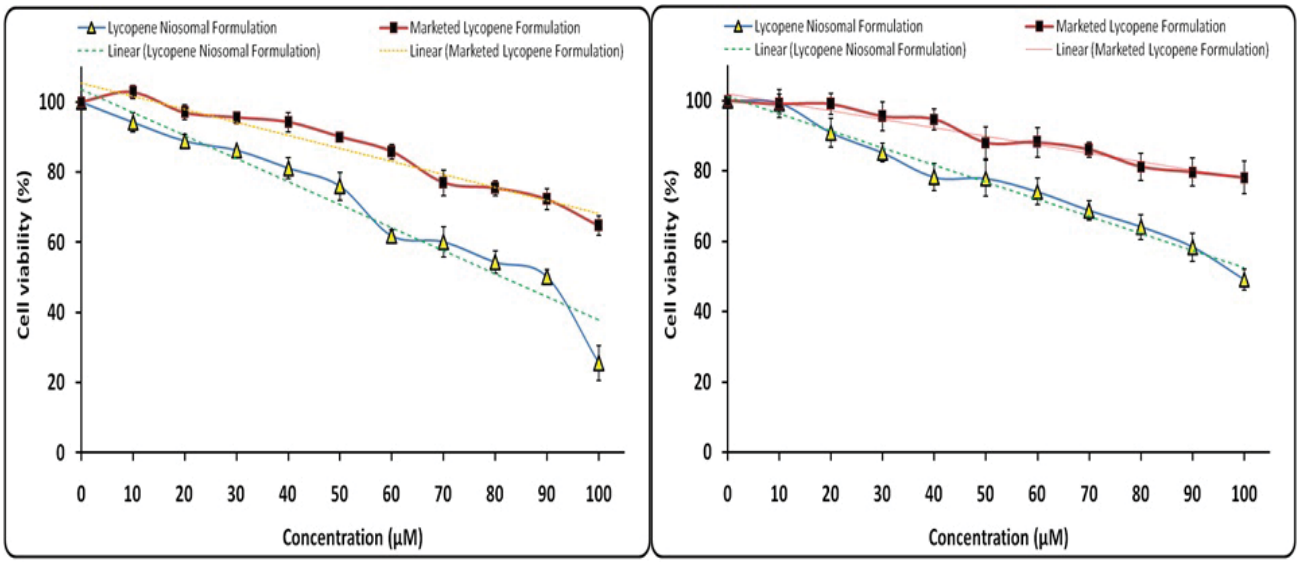

Figure 8: Graph showing anticancer activity of lycopene niosomes on cell lines, (a) Plot of concentration (µM) against cell viability profile in HeLa cell line, (b) Plot of concentration (µM) against cell viability profile in MCF-7 cell line.

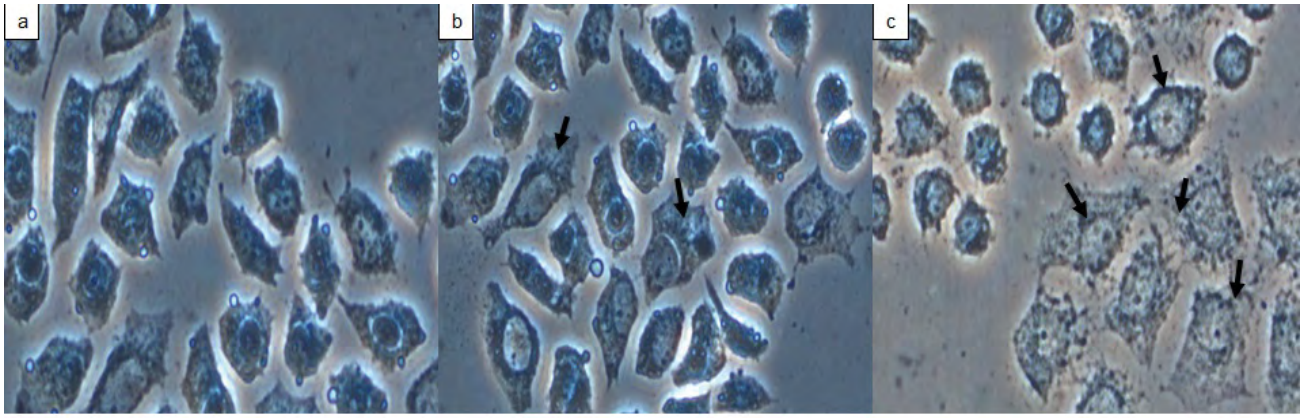

Figure 9: Confocal microscopic image of anticancer (HeLa cell line) activity showing apoptosis after 48 h (20X magnification), (a) Image of control cells, (b) Image of cancer cells when treated with marketed lycopene formulation (LYCORED™), (c) Image of cancer cells when treated with lycopene noisome.

the wall and core materials [28]. The adsorption-hydration method involves hydration of the lycopene adsorbed glass wool by hydrating media (mannitol) at required temperature of 60°C with agitation. The mannitol solution (pH 7.4) was added as an aqueous phase to impart isotonicity to the niosomal formulation. In niosomes structure, the lycopene was embedded within the bilayer of surfactant itself through hydrogen bond, thus was more protected against oxidative degradation, hence enhancing stability. The absence of clumps, agglomeration, and uniform consistency was observed in the prepared formulation, enhancing its shelf life. The sonication of lycopene adsorbed glass wool in hydration media helped in formulation of uniformly sized lycopene-loaded niosomes, due to uniform distribution of energy, forming uniform, unilamellar, and spherical outer structure. The maximum interaction of lycopene adsorbed in glass wool with the wall materials provides greater surface area for interaction. The reported average particle size of niosomes was excellent in circumventing the bio-environment and was found stable and avoids the uptake by rough endoplasmic reticulum. In order to study the modality of drug entrapment in lycopene nanoparticles, the effect of pH variations on zeta potential of both lycopene-loaded and un-loaded niosomal dispersions was studied. The zeta potential of empty nano-noisome has not shown any significant change in a different range of pH from 4 to 9, indicating the absence of ionization phenomena of the membrane components. Both unloaded and lycopene-loaded niosomes confirmed good stability over a three-month storage period. In fact, no statistically significant difference in size, zeta potential and entrapment efficiency of the empty vesicle were observed ($P>0.05$) after three-month storage at ambient temperature. Moreover, the reduction of drug encapsulation of this system was not significant with respect to the freshly prepared sample.

The *in vitro lycopene* release profile was gradual over time. Initial release phase of lycopene was faster when observed over a period of 10 h. This was possibly due to the incorporation of lycopene in the wall of niosomes. The release thereafter was steady and 67.3% of the entrapped lycopene was traced during the study period of 72 h. The mechanism of lycopene release from niosomal formulation was determined using the mathematical kinetic models and found to beFickian type and obeyed zero-order release kinetics, the regression equation was the best fit in Korsmeyer-Peppas model ($r^2=0.9915$) (Table 5). Thus, *in vitro* lycopene

release profile showed sustain and prolong release profile required for effective treatment, which might be due to maximum lycopene entrapment and uniform sized niosome particles prepared innovatively.

The bioavailability study of lycopene from nano-niosomes revealed a noticeable rise in blood plasma level. The relative bioavailability showed 297.19% increase (3-fold higher) in plasma lycopene level as compared to marketed formulation Lycored, which is expected due to control release, high entrapment efficiency and greater permeability of nano-niosomes. In general, the highly lipophilic drugs, such as lycopene, are supposed to be transported en route for the systemic circulation, via the TG absorption pathway, by associating with chylomicrons within enterocyte cells. The timing of the peak lycopene in plasma strongly favors the hypothesis that lycopene absorption is associated with the lipid absorption pathway. Interestingly, a distinct advantage of the lycopene nano-niosomes formulation compared to the Lycored product (Figure 7), which reflects the impact of the non-ionic surfactant and cholesterol used in the nano-niosomes which stimulates intestinal chylomicron production and promote absorption of lycopene.

The *in vitro* anticancer study was performed on MCF-7 and HeLacell lines [29-31] to determine the MSC and IC50 values. In both HeLa cells and MCF cells, the cell viability greatly decreased when the cells were incubated with the lycopene nano-niosomes, indicating that lycopene-loaded nano-niosomes are highly cytotoxic to the HeLa and MCF cells. The percentages of surviving HeLa cells and MCF cells obviously decreased with increasing concentrations when compared to marketed formulation Lycored and prolonged incubation time. These results indicated that the lycopene-loaded nano-niosomes play a key role in killing the HeLa and MCF cells as presented in Table 7. The effective inhibition of cell growth with lycopene-loaded nano-niosomes might be linked to the Fickian type drug release from the niosomes. The lycopene has the potential to induce apoptosis in tumor cells. In the present study, the apoptosis was clearly observed in the HeLacells when treated with lycopene-loaded nano-niosomes. These results demonstrated that the apoptosis of HeLa and MCF-7 was induced by lycopene released from the nano-niosomes and thus could be useful for various anticancer applications.

These results show the superiority of the method used for the preparation of lycopene niosome formulation in its stability, drug release and more importantly in preserving its *in vivo* and *in vitro* activity.

Conclusions

The lycopene niosome formulation prepared by adsorption-hydration method was found to be efficient and has preserved the lycopene's activity. This method promises to be a novel technique for enhancing entrapment efficiency by niosome formulation. The formulated nano-niosomes have potential to play a vital role in efficient herbal delivery of a broad spectrum of anticancer agents. The technique is simple and reproducible for further application, and could be useful for different therapeutic applications.

Acknowledgements

The author would like to thank Mr. Abhishek Tiwari (ICMR) for his support and suggestions during the study.

Conflict of Interest Disclosure

The authors Purnendu Kumar Sharma, Prachi Saxena, A. Balasubramaniama, A. Jaswanth, M. Chalamaiah, and Rakesh K. Tekade declare that they have no conflict of interest.

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