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6-shogaol from ginger oleoresin loaded liposomes using DMPG-Na as a carrier enhances the *in-vitro* and *in-vivo* anticancer activity

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ABSTRACT

Ginger oleoresin (GO) plays an important role on the attenuation of complications associated to the cancer therapy which is attributed to 6-shogaol (6-SGL). The major challenge in using 6-SGL for therapeutic applications is its poor aqueous solubility, low stability in GI and low bioavailability. Considering the potent anticancer nature of 6-SGL and its synergistic activity with other constituents in GO, there is a need to develop a suitable drug delivery system. Thus in the present study, 6-SGL rich GO (6-SRGO) was incorporated into liposomes by solvent injection technique using lipid (DMPG-Na) as carrier. The prepared 6-SRGO loaded liposomes (6-SRGO-LLPS) were characterized physically and chemically using FTIR, DSC, surface morphology, drug content (DC), encapsulation efficiency (EE), particle size (PS), zeta potential and further evaluated for stability study, *in vitro* release study, *in vitro* cytotoxicity study and *in vivo* anticancer activity in comparison with 6-SRGO. 3^2 factorial designs was used for optimization of formulation variables and the results of DC, EE and PS obtained were 89.92 ± 1.56, 79.36 ± 1.05 and 356.11 ± 4.07 respectively. Considering the above findings, 6-SRGO-LLPS exhibited significant *in vitro* (GI50- <10 μg/ml) and *in vivo* anticancer activity in comparison with 6-SRGO.

INTRODUCTION

Now a day's natural dietary foods including vegetables, fruits, and spices aliments have been achieved a great attraction from the scientist of various health care departments, food industries and the normal public because of their safety, efficacy and availability at the comparatively lower price. In addition they are believed to have no or very less toxic effects than the synthetic drugs (Kelly *et al.*, 2016; Shukla and Singh, 2007). However, the low aqueous solubility, poor bioavailability, and the stability of phytoconstituents lead to hamper their use in the treatment of various diseases. Therefore, various delivery system including nanotechnology-based drug-delivery systems have been designed for different naturally occurring phytoconstituents to increase the aqueous solubility, bio-availability and reduce the dose without hampering the efficacy of the drug, safety and the compatibility with patients (Bothiraja *et al.*, 2013).

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6-shogaol rich ginger oleoresin (6-SRGO) is an oleoresin extracted from *Zingiber officinale* Rosc., containing higher content of phenolic ketones; gingerols and shogaols (Oriania *et al.*, 2016; Singh *et al.*, 2008). Traditionally 6-SRGO used for the treatment of cough, stomach upset, inflammation, diarrhea, nausea and vomiting. Among the various phenolic constituents of ginger oleoresin (GO), the analogs of shogaols and gingerols are known for their potent antioxidant, anti-inflammatory, and anticancer activities (Chen *et al.*, 2012). Various studies have discovered that 6-shogaol (6-SGL) possesses most potent anti cancer activity than the other analogs of the shogaols and the gingerols (Wu *et al.*, 2010). Various *in vitro* studies have demonstrated the role of 6-SGL in suppressing the different cancer cells like ovary (Kim *et al.*, 2008), lungs, colon, gastrointestinal tract and neuroblastoma. The metabolites of 6-SGL are also known to possesses anticancer activity (Shukla and Singh, 2007; Surh *et al.*, 1998).

The major challenge of using 6-SGL is its poor aqueous solubility; which limits its therapeutic efficacy (Nikam *et al.*, 2013). High dose of 6-SRGO is required to reach the therapeutic efficacy, which may lead to the several side effects such as diarrhea, nausea and many times the tolerance of the

6-SRGO. There are few attempts have been made to develop nanoformulations using conventional extract of ginger but detail investigation, characterization and anticancer potential are not reported (Kumar *et al.*, 2011; Uthumpa *et al.*, 2013; Ratcharin *et al.*, 2012). Prompted by the above facts, present research aims at incorporating 6-SRGO within polymers to develop a drug delivery system displaying enhanced drug solubility, achieving site-specific tumor accumulation while reducing systemic toxicity of 6-SRGO.

Nanoparticular drug delivery systems using liposomes is an emerging technologies for the rational delivery of cytotoxic drugs in the treatment of cancer (Gao *et al.*, 2011; Li *et al.*, 2011; Tong *et al.*, 2012; Muthu *et al.*, 2012; Saxena and Hussain, 2012). Their use offers solubilization of water insoluble drugs, high drug loading, improved stability, increases circulation time, improved tissue targeting, control and extended drug release and lower systemic toxicity (Malam *et al.*, 2009). Liposomes are closed spherical vesicles consisting of a lipid bilayer that encapsulates poorly water soluble drugs into the inner hydrophobic core thus improving solubility, stability and bioavailability (Akbarzadeh *et al.*, 2013). Due to its outer hydrophobic shell and nano size particles they sometimes evince prolonged circulation times *in vivo* and can hoard in tumoral tissues. The liposomes size varies from 200-400 nm adduce rival physical and chemical properties that can be exploited for drug delivery by conjugation with drugs. The commercial availability of liposomal Doxil and albumin-nanoparticle-based Abraxane has existed for the treatment of the cancer. Due to above applications, liposomes offer increased precision in chemotherapeutic targeting of breast cancer.

To the best of our knowledge no previous studies have attempted the preparation of 6-SRGO-loaded liposomes (LLPS) to improve its anticancer efficacy. Therefore, this study was attempted to investigate the potentials of 6-shogaol rich ginger oleoresin-loaded liposomes (6-SRGO-LLPS), which may enhance solubility, physiological stability and anticancer efficiency of 6-SRGO.

MATERIALS & METHODS

Carrier and core materials

Liposomes were produced using a carrier 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-glycerol, sodium salt, (DMPG-Na)lipid that was supplied by Lipoid, Cologne, Germany, with melting point at 122°C. The reference standard (pure isolated) 6-shogaol (>96% purity w/w) was procured from Natural Remedies Pvt. Ltd. Bangalore, Karnataka, India. 6-SRGO was purchased from Nisarg Biotech, Satara, Maharashtra, India. Dialysis bags (MW cut-off 12,000) were purchased from Sigma-Aldrich Chemical Private Ltd (Bangalore, India). All other reagents and chemicals used in this study were of analytical grade and procured from Merck Specialties Private Limited, Mumbai, India.

Quantification of 6-SGL present in GO

6-shogaol present in ginger oleoresin was quantified using RP-HPLC method which was reported in our earlier study (Nikam *et al.*, 2013).

Preparation of 6-SRGO-LLPS

6-SRGO-LLPS were prepared according to solvent injection technique (Laouini *et al.*, 2012). Extraction of 6-SGL was performed by adding the ginger oleoresin to a chloroform containing a specific amount of DMPG-Na followed by mixing with a suitable quantity of cholesterol under stirring at room temperature. To the sufficient distilled water, above mixture was added rapidly under magnetic stirring at 2000 rpm for 45 min followed by sonication for 10 min to obtain liposome suspension. Blank liposomes (devoid of 6-SRGO) were prepared in the similar manner.

Optimization of 6-SRGO-LLPS by 3^2 factorial designs

3^2 factorial designs was adopted to optimize the liposomes composition while studying the effect of lipid and cholesterol (independent variables) on Drug Content (DC), Entrapment Efficiency (EE) and Particle Size (PS) (three dependent variables or responses) by obtaining a response surface plot.

Characterization of 6-SRGO-loaded liposomes

Liposomes size distribution & zeta potential

The size of blank and 6-SRGO-LLPS was determined by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The particle size measurements were carried out at a 90° scattering angle. The samples were dispersed in distilled water and the average PS was determined and expressed in terms of d(0.9) μm. The zeta potential was measured with the laser doppler electrophoretic mobility measurements using Zetasizer 3000 (Malvern Instruments) at a temperature of 25°C.

DC & EE

The concentration of 6-SGL in the liposomes was determined by rapid and sensitive RP-HPLC method. The superficial 6-SGL was determined by washing 6-SRGO-LLPS with ethanol and measuring the 6-SGL content collected in the washing solution. EE was determined as the ratio between the encapsulated 6-SGL (total — superficial) and the feeding amount of 6-SGL (active compound). The DC was determined as the ratio between the mass of liposomes collected at the end of the process and the mass of carrier added to 6-SRGO used in the production of liposomes.

Physical characterization

Fourier transform infrared spectroscopy

The pure 6-SRGO, DMPG-Na and 6-SRGO-LLPS were characterized by FTIR spectroscopy in the 4000 to 400 cm^{-1} region using FTIR Spectrophotometer (Jasco 4100).

Differential scanning calorimetry

Thermal analyses were performed by differential scanning calorimetry (DSC 821, Mettler Toledo). Approximately 5 mg of 6-SRGO-LLPS were heated in hermetically sealed alluminium pan with heating rate 10°C per min under nitrogen atmosphere (flow rate 50 ml/min). The pure 6-SRGO was also analyzed for comparison.

Surface morphology

The morphology of 6-SRGO-LLPS was performed using transmission electron microscopy (TEM). The sample preparation for the TEM was followed by (Bothiraja *et al.*, 2013).

Evaluation of 6-SRGO-LLPS stability

6-SRGO-LLPS were transferred in glass vials with plastic lids, protected from the light, and stored at 25°C for 90 days for short term stability studies. Liposomes were evaluated with regard to the content of 6-SGL, PS and EE.

In vitro release of 6-SRGO from liposomes

The *in vitro* release of 6-SRGO-LLPS was carried out in 0.1 N HCL (pH 1.2) and in phosphate-buffer (pH 7.4) using dialysis bag diffusion technique. A formulation equivalent to 1 mg of 6-SRGO or 1 mg 6-SRGO solution (1 mg/ml in 50% w/w mixture of PEG 400 and water) as control was introduced into a dialysis bag (cellulose membrane, MW cut-off 12,000 Da), sealed and immersed into 50 ml of release medium. The temperature of entire system was maintained at 37 ± 0.5°C with continuous magnetic stirring at 100 rpm. At previously planned time intervals, the sample was removed and replaced with an equal volume of fresh medium in order to maintain sink conditions. 6-SGL present in the solution was quantified by using rapid and sensitive RP-HPLC method. Analysis of data was performed using PCP Disso software (V3; Poona College of Pharmacy, Pune, India).

In vitro cytotoxicity study

In vitro cytotoxicity study of free 6-SRGO, blank liposomes and 6-SRGO-LLPS were evaluated against human breast cancer MCF-7 using *in vitro* SRB assay at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai. The results of MCF-7 were compared with that of marketed Adriamycin (Doxorubicin). The cytotoxicity protocol for SRB assay was followed by method described by (Kaushik *et al.*, 2015).

Acute toxicity study

The acute toxicity study was performed for 6-SRGO and 6-SRGO-LLPS on swiss albino mice. The animals were randomly divided into eleven groups (n = 6). The first group (control group) received feed and distilled water orally. Groups 2–6 were orally treated with 6-SRGO with doses 55 mg/kg, 175 mg/kg, 550 mg/kg, 1750 mg/kg and 2000 mg/kg body weight, respectively. Groups 7–11 were orally treated with 6-SRGO-LLPS with doses 55 mg/kg, 175 mg/kg, 550 mg/kg, 1750 mg/kg and 2000 mg/kg body weight, respectively. The animals were continuously observed for general behavioral changes, sign of toxicity, and mortality for 1 h after treatment and then intermittently for 4 h and thereafter over a period of 24 h. Mice were further observed for up to 14 days for behavioral change and sign of toxicity.

In vivo anti cancer activity of 6-SRGO and 6-SRGO-LLPS against Dalton's Ascitic Lymphoma (DAL) in mice

Selection grouping and acclimatization of laboratory animal

Male Swiss albino mice having the weight range of 20 gm to 25 gm were used to conduct this study. They were kept in to micro nylon boxes at control temperature (temp 25 ± 2°C).

"The protocol of the study was approved by Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (approval number CPCSEA/Q.A./01/2014-15; institutional approval no- CPCSEA/1999/100). The Declaration of Helsinki as amended in Seoul 2008 for humans, and the European Community guidelines as accepted principles for the use of experimental animals, was followed."

Induction of cancer cells

DLA cells were procured from Amala cancer research center, Trissur, Kerala, India. The cancer cells implants *in vivo* in mice by intraperitoneal route. Before transferring the cancerous cells to mice, by using saline solution the DLA cells were taken from peritoneal cavity of the mice. The total cells count was maintained up to 1×10^6 after dilution and was given intraperitoneally. The treatment was started after seven days of induction of tumor cells (Sathiyanarayanan *et al.*, 2006; Unnikrishnan and Kuttan, 1990).

Grouping of the mice and outline of the treatment

Animals were grouped in to nine groups with each group contains six animals. The animals of eight groups have given DLA cells (1×10^6 cells per mice) intraperitoneally, and one group is remains as a control group.

Group 1 and **2** represents normal control and tumor control respectively.

Group 3 represents positive control, (Received 5-fluoro uracil injection at 20 mg/kg body weight)

Group 4, 5, and 6 represents treatment control, (Received 6-SRGO at a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight respectively through orally).

Group 7, 8, and 9 represents treatment control group, (Received 6-SRGO-LLPS at a doses of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight respectively through orally).

Evaluation of clinical parameters

During this study, the cancer cells were inoculated in to the animals and the treatment for cancer was started after 24 hrs once a day for 14 days. Once after all the doses were finished, animals were sacrificed by euthanasia. The samples of the blood were withdrawn from each mouse at predetermined time from retro orbital plexus bleeding. Various parameters were checked which include, count of the cancer cells, hematological parameters consist of content of Hb, RBC count, WBC count, platelet count and the volume of packed cells, serum enzyme and lipid profile including count of total cholesterol (TC), triglycerides (TG), aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) and the derived parameters such as total life span (%) of the animal, body weight of the animal used and the cancer cell count.

Statistical analysis

All data are expressed as means ± SEM. One way ANOVA followed by Dunnettspost test was used for *in vivo* anticancer activity. P < 0.01 was considered as significant. Prism 5 Demo software has been used for the statistical analysis.

RESULTS AND DISCUSSION

6-SGL is well known for its anticancer potential. However its usage is limited due to its low solubility, low stability

in GI and low bioavailability. GO consists of higher amount of shogaols and gingerols compare to normal solvent extracts. Hence usage of GO is ideal for anticancer purposes especially due to the presence of higher amount of 6-SGL. Further, synergistic effect of herbals is always plays a role in therapeutic applications. Although the pharmacological activities are attributed to active constituents, practically isolation and formulation of pure compounds from herbals are expensive processes and there are possibilities of reduction in therapeutic activity due to lack of synergism while using single compound. So it is worthwhile to develop drug delivery systems for multi chemical compositions from herbals rather than single compound. Considering these facts in present study, 6-SRGO-LLPS have been developed and investigated as nanocarriers for oral administration in order to enhance both *in vitro* and *in vivo* anticancer activity of 6-SGL in GO.

Quantification of 6-SGL present in ginger oleoresin

In the present study 6-SGL present in the GO was quantified by rapid and sensitive RP-HPLC method and the 6-SGL was quantified at retention time of 10.30 min at the wavelength 281 nm (Figure 1). The amount of 6-SGL present in 6-SRGO was found to be 20% ± 2% where as the optimized formulation 6-SRGO-LLPS was found to have 16% ± 1%. The reduction in the amount of 6-SGL in the optimized formulation is due to the drug loss during the preparation of liposomes.
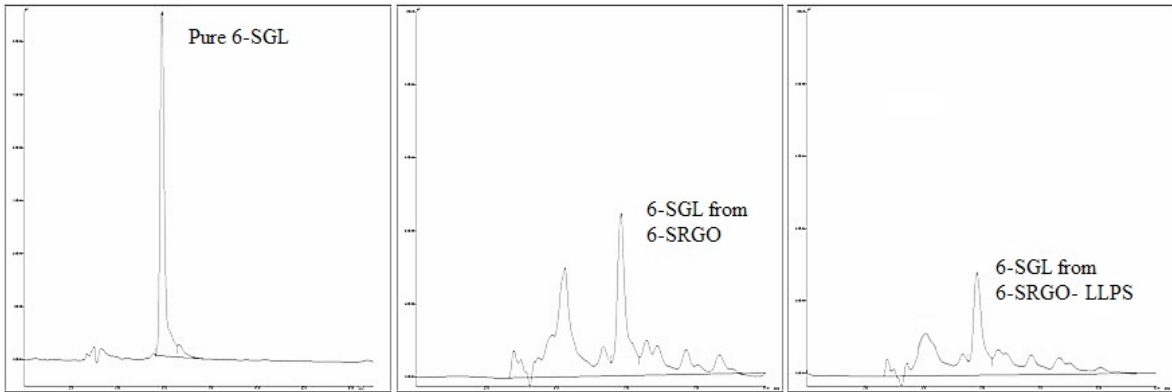

Fig. 1: HPLC chromatograms of pure 6-SGL (A), 6-SGL from 6SRGO (B) and 6-SGL from 6-SRGO-LLPS (C) formulation.

Preparation and optimization of 6-SRGO-LLPS by 3^2 factorial designs

6-SRGO-LLPS was prepared and the effect of these liposomes composition on DC, EE and PS was studied by 3^2 factorial designs (Shukla and Singh, 2007). The mean PS was observed in between 250–450 nm and was affected by the selected variables (r^2 = 0.9247). The DC and EE were in the range of 79–96% and 72–88% respectively. A good fit (r^2 for DC = 0.8580 and EE = 0.9236) was observed between the DC, EE and the independent variables.

A series of 6-SRGO-LLPS were prepared and observed physically for phase separation. In process of preformulation study, the concentration of DMPG-Na and cholesterol that would give non-coagulation and non-sedimenting liposomes were determined. A 3^2 factorial design was taken to optimize their concentrations. The concentration of 6-SRGO was kept constant. As per 3^2 factorial designs, nine different batches of different concentration ratio of lipid and cholesterol were prepared. The responses obtained from these batches are shown in Table 1. The data obtained was subjected to multiple regression analysis using Design Expert 10 software.

The results of multiple regression analysis of the obtained data are summarized in Table 1. The adequacy of fitted model was checked by analysis of variance. To study the interaction effects of the independent variables, response surface plots were constructed using Design Expert 10 software (Figure 2).

From the factorial design study (Table 1), it is observed that positive coefficients of the main term X1 and the interaction term X1X1 indicated a favorable effect on the mean liposomes size, with the lipid chiefly influencing than cholesterol. The lipid had a linear effect on the mean liposomes size as seen in surface plot (Figure 2A). Smaller liposomes were obtained at low lipid content, probably due to high distribution efficiency of internal phase into external phase. Increase in the viscosity of internal phase with increased amount of lipid also provides resistance for mass transfer during diffusion of internal phase into the external phase leading to liposomes enlargement. The increase in the mean liposomes size may also be due to hydrophobic-hydrophobic interaction of lipid with the drug.

Table 1: Regression analysis results of measured responses.

Coefficients	Parameters		
	Particle size	Drug content	Encapsulation efficiency
β0	356.11	89.92	79.36
β1	50.33	+1.79	+5.21
β2	12.67	+1.58	+5.58
β11	-	-0.62	-1.42
β22	-	-0.50	-1.17
β12	-	+0.12	-0.38
r²	0.9247	0.8580	0.9236

Both X1 and X2 showed favorable positive effects on DC and EE. In case of lipid it showed linear effect on DC and EE. Further more in case of cholesterol, it also showed linear effects on DC and EE. As seen in surface plot (Figure 2B & 2C), the lipid favored DC and EE by hydrophobic-hydrophobic interactions

with drugs leading to formation of interpenetrated network chain. However, cholesterol exerted an opposite effect as it led to

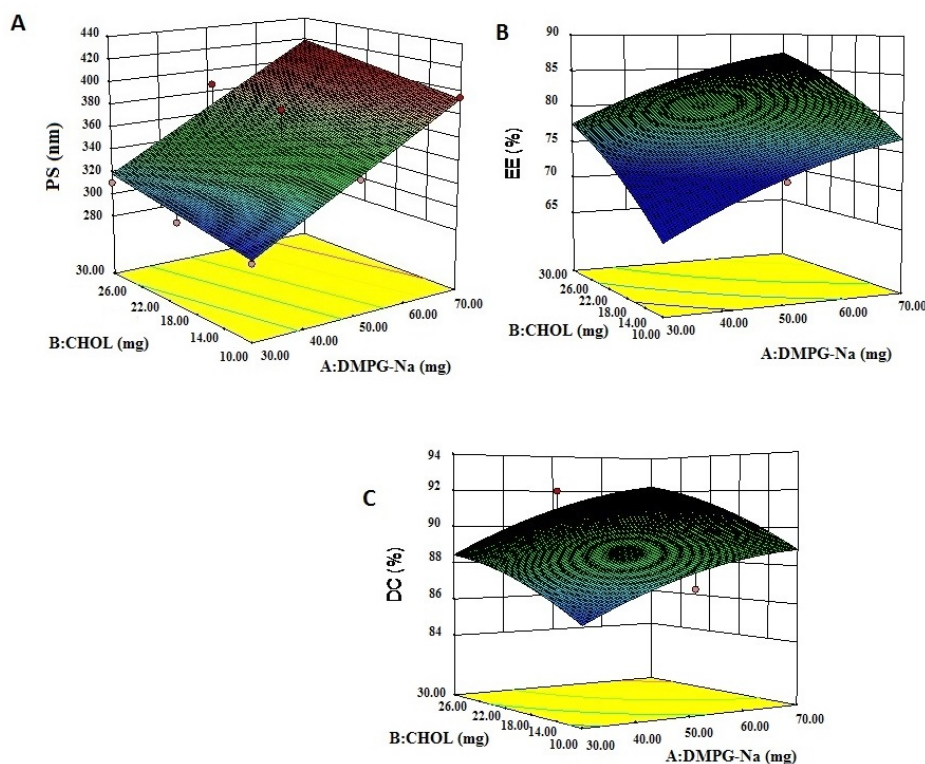solubilization of the drug in the external phase.

Fig. 2: Response surface plot illustrating effect of factorial variables. (A) Particle size, (B) Encapsulation Efficiency, (C) Drug Content.

Based on the results of the factorial designs, 6-SRGO-LLPS having acceptable PS, DC and EE was selected as an optimized batch. The size of the optimized 6-SRGO-LLPS was 380 ± 3 nm – which was not significantly different from its blank counter part. Large molecules of more than 40 kDa in size and certain particles ranging from 10 to 400 nm can leave the vascular bed and accumulate inside the interstitial space of the tumor (EPR effect) (Gao *et al.*, 2011). Drug delivery to specific sites of the body is influenced by size of the liposomes; smaller liposomes may tend to minimize the particle uptake by non targeted cells, including their premature clearance by the mononuclear phagocytic system (Brigger *et al.*, 2002). It is hypothesized that liposomes developed in the present study are of appropriate size to be able to passively target the tumor site.

Physical characterization

Fourier transform infrared spectroscopy

6-SRGO, DMPG-Na, and 6-SRGO-LLPS were analyzed by Fourier transform infrared (FTIR) spectroscopy and the results are showed in (Figure 3).

In the IR spectra (Figure 3A) of 6-SRGO following peaks were assigned, -OH stretching ($\sim$3124 cm^{-1}), Aromatic –CH stretching ($\sim$2994 cm^{-1}), Aliphatic –CH stretching ($\sim$2852 cm^{-1}), –C=O stretching ($\sim$1736 cm^{-1}) and OH bending out of plan ($\sim$1373 cm^{-1}), while in the formulation (Figure 3C), 6-SRGO incorporated with polymer lipid, remarkable peaks of active drug

such as –OH stretching at ($\sim$3124 cm^{-1}) were not observed. Thus final IR spectra of 6-SRGO-LLPS revealed that 6-SRGO has been completely encapsulated with lipid in the formulation.

Differential scanning calorimetry

Differential scanning calorimetry was performed with 6-SRGO-LLPS to determine the onset, maximum temperature and enthalpy of 6-SRGO-LLPS and 6-SRGO (Figure 4). According to the results, melting point of free 6-SRGO was observed at around 240°C with the enthalpy 31.11 J/g. In case of 6-SRGO-LLPS thermogram, the free 6-SRGO peak was disappeared and shifted to 169°C indicating molecular dispersion of 6-SRGO inside liposomes. Thermograms of 6-SRGO-LLPS exhibited a sharp endothermic peak starting at approximately 145°C and ending at approximately 185°C. Onset temperature at approximately 145°C and melting point around 169°C are desirable for particle stability at room or lower temperatures and digestibility in the gastrointestinal tract, respectively, acting as a trigger to release the active core during the digestion process (Tulini *et al.*, 2016).

Surface morphology

The obtained 6-SRGO-LLPS solution appeared clear and light yellowish in color. Figure also revealed that the prepared formulation was monodispersed in water phase, tightly packed and spherical shaped liposomes whose size measured by laser scattering technique was correlated well with that by TEM (Figure 5).
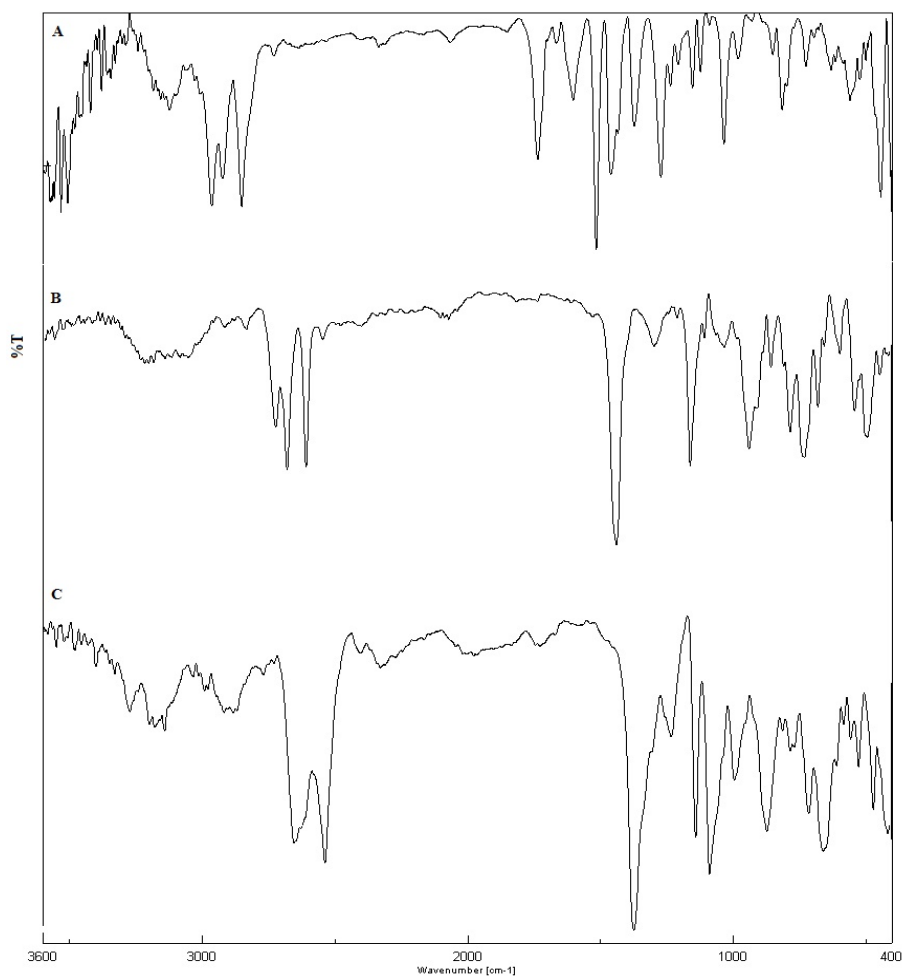
Fig. 3: Fourier transform infrared (FT/IR) spectroscopy analyses of (C) 6-SRGO-LLPS, compared with (A) 6-SRGO and (B) Phospholipid.
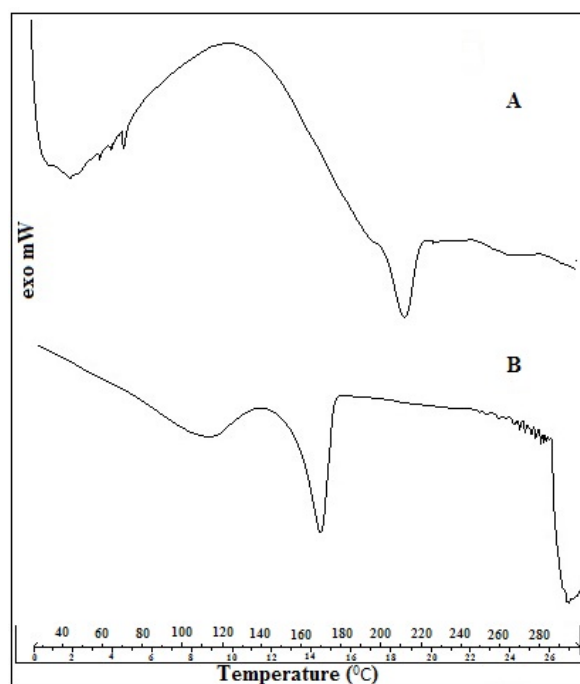

Fig. 4: Differential scanning calorimetry (DSC) analyses of (A) 6-SRGO and (B) 6-SRGO-LLPS.
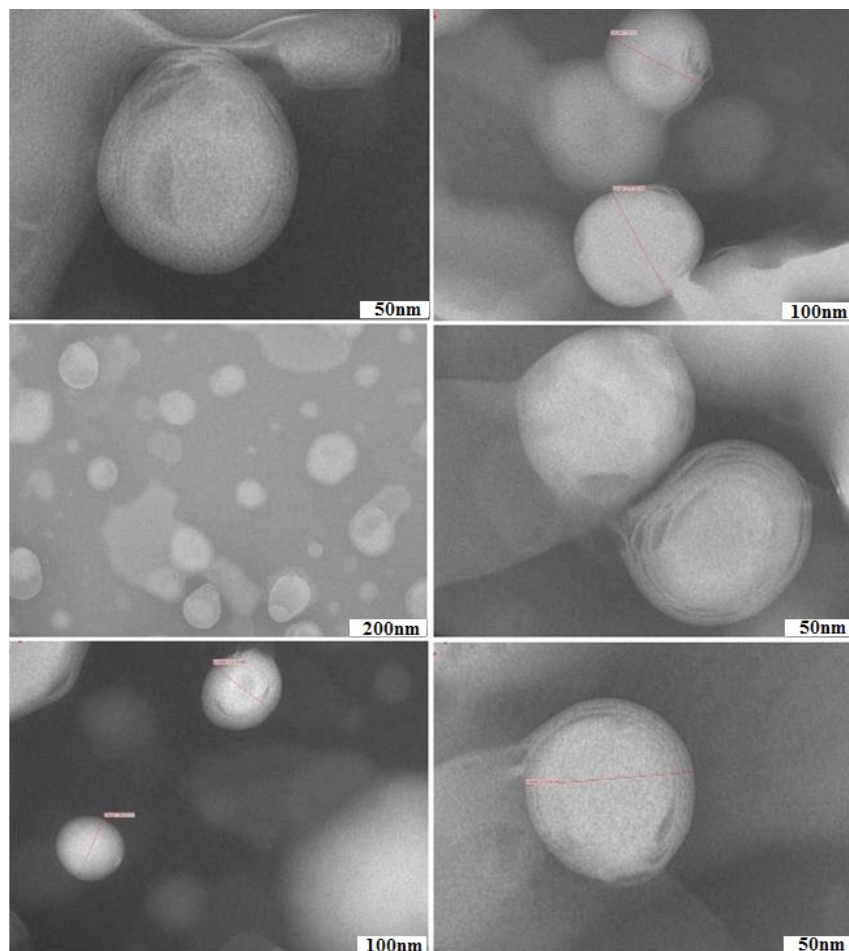
Fig. 5: Transmission electron micrograph of optimized 6-SRGO-LLPS.

The TEM image showed that there is no drug crystallization appears in the 6-SRGO-LLPS and the surface of the liposomes are smooth and they are suspended uniformly in the formulation.

Evaluation of 6-SRGO-LLPS stability

Stability study reveals that 6-SRGO-LLPS did not show noticeable changes in their size (340 ± 15 nm) and EE (79 ± 2.01%). The sedimentation of the drug was not observed during short-term stability studies. A drug loss of meager 3.62% was probably due to the partial decomposition of 6-SGL. These indicated that the drug-loaded liposomes were physically stable at room temperature for at least 3 months.

In vitro release study

The dialysis method is the most suitable method that simulated the circumstance of drug *in vivo*. The release pattern of 6-SRGO-LLPS in comparison with 6-SGL from 6-SRGO was investigated in gastric fluids (pH 1.2) and intestinal fluids (pH 7.4). Figure 6 showed the dissolution profile of 6-SGL from 6-SRGO-LLPS and 6-SRGO in both HCl (pH 1.2) and phosphate buffer saline (pH 7.4), respectively. 6-SGL release from liposomes was always slower than the corresponding 6-SGL from 6-SRGO which escaped rapidly from the dialysis bag approximately 60% within 2 h and the release was over 95% by 6 h. The profile of the 6-SRGO-LLPS showed a very slow release of 6-SGL approximately 30%
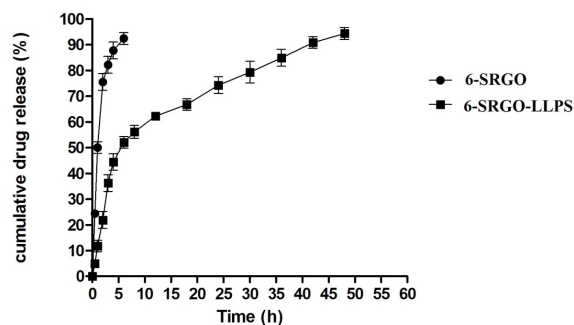
within 2 h and the release was over 90% by 42 h.

Fig. 6: *In vitro* release profiles of 6-SGL from 6-SRGO and from 6-SRGO-LLPS.

The drug release rate between liposomes at both pH ranges was tested. The 6-SGL from 6-SRGO exhibited faster release than the 6-SRGL-LLPS at all the pH ranges evaluated. The 6-SGL release behavior reflected the 6-SGL incorporation stability in the liposomes. The drug incorporated into the inner core compartment stayed tightly inside the liposomes. The results suggested that pH had little effect on the dissolution of 6-SGL from the liposomes, while 6-SGL release markedly increased with increasing pH value. The reason may be that as an, 6-SGL is

unstable in low pH i.e. in gastric condition, which may lead to a   low bioavailability of 6-SGL from 6-SRGO.
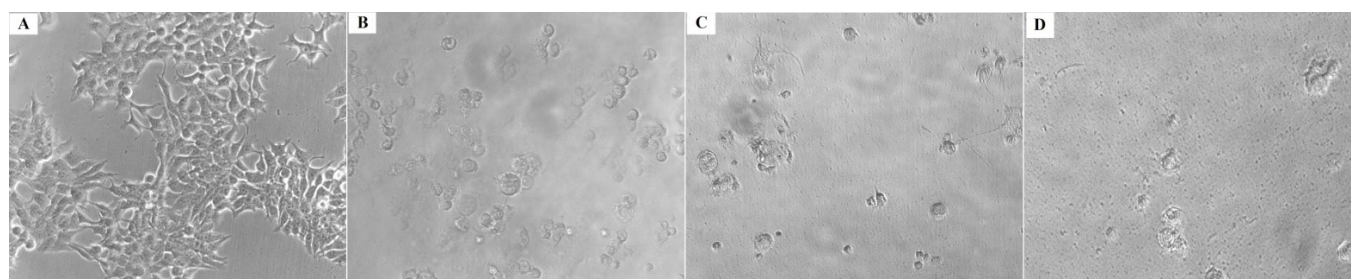

Fig. 7: *In vitro* cytotoxicity study on breast cancer cell line (MCF 7), (A) MCF 7 control, (B) Treatment control, (C) Treated with 6-SRGO, (D) Treated with 6-SR-GO-LLPS.

Table 2: *In vitro* anticancer effect of 6-SRGO-LLPS on breast cancer cell MCF-7.

MCF 7	Drug concentration (μg/ml) calculated from graph		
	LC50	TGI	GI50
6-SRGO	>80	56.4	26.8
Blank	>80	>80	>80
6-SRGO-LLPS	>80	38.4	<10
ADR	60.8	29.5	<10

Each point represents an average ± SD (n = 3).

In vitro cytotoxic activity

The *in vitro* cytotoxic activity of 6SRGO-LLPS was investigated and compared with free drug in solution, blank liposomes and marketed Adriamycin (Doxorubicin) against human breast cancer MCF-7 cells using *in vitro* SRB assay. The LC50, TGI and GI50 values were illustrated in Table 2, indicated that 6SRGO-LLPS displayed better cytotoxic activity than the 6-SRGO and the blank liposomes. The GI50 concentration against MCF-7 was found to be <10 μg/ml, 26.8 μg/ml and >80 μg/ml for 6-SRGO-LLPS, 6-SRGO and blank liposomes respectively (Fig. 7).

Table 3: Effect of 6-SRGO and 6-SRGO-LLPS on Hematological parameters.

TREATMENT	Total WBC	Rbc Count	Hb	PCV %	Platelets
	Cells/ml × 10³	Mill/cumm	gm/dl		Lakhs/cumm
G_1	10.35 ± 1.30	4.30 ± 1.85	12.50 ± 1.34	14.25 ± 2.44	3.30 ± 0.95
G_2	15.22 ± 2.64[a**]	2.68 ± 0.72[a**]	6.80 ± 0.95[a**]	38.36 ± 3.35[a**]	1.70 ± 0.42[a**]
G_3	12.32 ± 1.30[b**]	4.05 ± 1.40[b**]	11.90 ± 1.48[b**]	16.40 ± 1.40[b**]	2.94 ± 0.65[b**]
G_4	12.90 ± 2.04[b**]	3.45 ± 1.05[b**]	11.60 ± 1.22[b**]	17.34 ± 2.30[b**]	2.88 ± 0.54[b**]
G_5	13.08 ± 2.26[b**]	3.85 ± 1.34[b**]	12.35 ± 1.66[b**]	18.08 ± 2.66[b**]	3.04 ± 0.68[b**]
G_6	12.66 ± 2.28[b**]	4.10 ± 1.45[b**]	12.15 ± 1.36[b**]	17.84 ± 2.46[b**]	2.94 ± 0.62[b**]
G_7	11.74 ± 1.38[b**]	4.25 ± 1.82[b**]	12.40 ± 1.64[b**]	17.18 ± 2.26[b**]	3.40 ± 0.90[b**]
G_8	11.66 ± 1.30[b**]	4.30 ± 1.85[b**]	12.44 ± 1.68[b**]	16.70 ± 2.18[b**]	3.46 ± 0.92[b**]
G_9	11.58 ± 1.26[b**]	4.35 ± 1.88[b**]	12.55 ± 1.74[b**]	16.65 ± 1.98[b**]	3.54 ± 0.95[b**]

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 to G_6 – Treatment control (6-SRGO 100, 200, 400 mg/kg), G_7 to G_9 – Treatment control (6-SR-GO-LLPS 100, 200, 400 mg/kg).
All values are expressed as mean ± SEM for 6 animals in each group and results were analyzed by using One way ANNOVA, followed by Dunnettspost test.
[**a]Values are significantly different from normal control (G_1) at $P < 0.01$.
[**b]Values are significantly different from cancer control (G_2) at $P < 0.01$.

The enhanced cytotoxic activity of 6-SRGO-LLPS may be attributed to greater cellular uptake of liposomes via phagocytosis or the fusion process of lipid liposomes. Therefore, 6-SRGO-LLPS might have served as a potential nanocarrier to improve the *in vitro* cytotoxic activity of 6-SGL. The lower cytotoxic activity of free drug in solution, blank liposomes may be due to its efflux by P-glycoprotein pumps.

Acute toxicity study

No death or toxic effect on tested animals were recorded during first 24 h as well as 14 days of observation after oral treatment of 6-SRGO and 6-SRGO-LLPS at the doses of 55 mg/kg, 175 mg/kg, 550 mg/kg, 1750 mg/kg and 2000 mg/kg body weight.

The present study showed that oral administration of 6-SRGO and 6-SRGO-LLPS in acute dose up to 2000 mg/kg body weight did not produce any sign of toxicity or death in mice, suggesting a lethal dose 50% (LD50) above 2000 mg/kg. An acute toxic study provides a guideline for selecting doses for *in vivo* study (1/10th and 1/20th of maximum dose in mice) which may be more clinically relevant (Mehta *et al.*, 2009). Thus, derived doses of 100 mg/kg, 200 mg/kg and 400 mg/kg for both 6-SRGO and 6-SRGO-LLPS have been selected.

Table 4: Effect of 6-SRGO and 6-SRGO-LLPS on serum Enzymes and lipid proteins.

Treatment	Cholesterol	TGL	AST	ALT	ALP
	(mg/dl)	(mg/dl)	(U/L)	(U/L)	(U/L)
G_1	110.085 ± 3.05	136.85 ± 2.55	38.40 ± 1.65	33.28 ± 1.45	132.28 ± 2.40
G_2	146.95 ± 4.34[a**]	220.28 ± 4.40[a**]	78.6 ± 2.74[a**]	62.32 ± 2.60[a**]	265.30 ± 4.35[a**]
G_3	126.30 ± 3.84[b**]	169.15 ± 2.65[b**]	44.40 ± 1.72[b**]	34.52 ± 1.70[b**]	154.45 ± 2.40[b**]
G_4	123.44 ± 3.64[b**]	172.32 ± 2.84[b**]	50.15 ± 2.82[b**]	40.35 ± 2.02[b**]	171.85 ± 2.90[b**]
G_5	122.30 ± 3.60[b**]	166.15 ± 2.56[b**]	49.05 ± 2.32[b**]	36.84 ± 1.86[b**]	172.35 ± 2.94[b**]
G_6	114.20 ± 3.52[b**]	171.80 ± 2.76[b**]	46.50 ± 2.20[b**]	37.35 ± 1.94[b**]	167.34 ± 2.36[b**]
G_7	112.45 ± 3.26[b**]	157.30 ± 2.45[b**]	41.22 ± 2.16[b**]	34.24 ± 1.40[b**]	158.26 ± 2.16[b**]
G_8	111.42 ± 3.24[b**]	149.22 ± 2.40[b**]	40.85 ± 2.10[b**]	33.60 ± 1.28[b**]	154.30 ± 2.14[b**]
G_9	110.65 ± 3.22[b**]	146.44 ± 2.34[b**]	39.60 ± 2.05[b**]	32.58 ± 1.24[b**]	152.28 ± 2.05[b**]

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 to G_6 – Treatment control (6-SRGO 100, 200, 400 mg/kg), G_7 to G_9 – Treatment control (6-SRGO-LLPS 100, 200, 400 mg/kg).
All values are expressed as mean ± SEM for 6 animals in each group and results were analyzed by using One way ANNOVA, followed by Dunnettspost test.
[**a]Values are significantly different from normal control (G_1) at $P < 0.01$.
[**b]Values are significantly different from cancer control (G_2) at $P < 0.01$.

In vivo anti cancer activity of 6-SRGO and 6-SRGO-LLPS against Dalton's Ascitic Lymphoma (DAL) in mice

The life span of the animal after starting the treatment for the cancer is the most reliable criteria to find out the efficacy of the drug used to treat the cancer (Clarkson *et al.*, 1965). In this study, the average life span of tumor control group of animals was found to be 48% where as an increase in life span was observed up to 78%, 81% and 84% for group treated with 6-SRGO at the doses of 100, 200, and 400 mg/kg body weight and 91.5%, 92.5% and 93.5% for group treated with 6-SRGO-LLPS at the doses of 100, 200 and 400 mg/kg body weight respectively. However, in group treated with 5-FU the overall life span of the animals was found to be 94% which shows very potent cytotoxic in nature.

As shown in Table 3, except the WBC count, all other hematological parameters have been decreased significantly for groups treated with 6-SRGO-LLPS than the groups treated with 6-SRGO at all three doses, compared to normal control group of animals.

The induction of DLA cells, rises the level of cholesterol, aspartate amino transferase, alanine amino transferase and alkaline phosphatase to the significant level when compared to the normal group. The treatment with 6-SRGO-LLPS recovers the above mentioned changes to normal level than the group treated with 6-SRGO at all three doses (Table 3).

Packed cell volume as well as viable tumor cell count were reduced in group treated with 6-SRGO-LLPS more significantly than the groups treated with 6-SRGO at all three doses when compared to tumor control groups (Tables 4 and 5).

In DLA tumor, a rise in ascitic tumor volume was noticed. Ascitic fluid gives rise to a direct nutritional source for tumor cells and a sudden rise in ascitic fluid with tumor growth to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994). Treatment with 6-SRGO-LLPS significantly reduces the increased level of ascetic fluid to the normal level when compare to the 6-SRGO at all three doses.

Usually, the major problem in the treatment of the cancer is the suppression of myeloma and the anemia (Price and Greenfield, 1958; Hogland, 1982). The anemia arises mainly because of reduction in the RBC count or reduction in the total hemoglobin count (Fenninger and Mider, 1954). Treatment with

6-SRGO and 6-SRGO-LLPS at all three doses brought backs all the hematological parameters to the normal level significantly.

Table 5: Effect of 6-SRGO and 6-SRGO-LLPS on the life span, body weight and cancer cell count of tumor induced mice.

Treatment	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml × 10^6
G_1	6	>30 days	2.22 ± 0.68	-
G_2	6	48%	9.44 ± 1.86[a**]	2.75 ± 0.80[a**]
G_3	6	94%	5.66 ± 0.42[b**]	1.30 ± 0.22[b**]
G_4	6	78%	5.98 ± 0.84[b**]	1.66 ± 0.50[b**]
G_5	6	81%	6.05 ± 0.88[b**]	1.38 ± 0.23[b**]
G_6	6	84%	6.18 ± 0.94[b**]	1.54 ± 0.36[b**]
G_7	6	91.5%	5.45 ± 0.55[b**]	1.34 ± 0.18[b**]
G_8	6	92.5%	5.22 ± 0.32[b**]	1.33 ± 0.15[b**]
G_9	6	93.5%	4.90 ± 0.20[b**]	1.32 ± 0.10[b**]

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 to G_6 – Treatment control (6-SRGO 100, 200, 400 mg/kg), G_7 to G_9 – Treatment control (6-SRGO-LLPS 100, 200, 400 mg/kg).
All values are expressed as mean ± SEM for 6 animals in each group and results were analyzed by using One way ANNOVA, followed by Dunnettspost test.
[**a]Values are significantly different from normal control (G_1) at $P < 0.01$.
[**b]Values are significantly different from cancer control (G_2) at $P < 0.01$.

Treatment with 6-SRGO-LLPS increased the life span of the mice, controlled the tumor volume and reduced the cell count of the tumor and normalized the hematological parameters more significantly than the group treated with 6-SRGO at all three doses. Thus the result shows that the 6-SRGO-LLPS exhibited better anticancer activity against DAL bearing mice as compare to the 6-SRGO at all three doses.

CONCLUSION

We have developed and investigated liposomes composed of lipids (DMPG-Na) and cholesterol as an effective nano carrier for the delivery of a natural lipophilic anticancer bioactive 6-SGL from 6-SRGO. The developed 6-SRGO-LLPS exhibited higher DC and sustained release of 6-SGL from 6-SRGO. Liposomal encapsulation of 6-SRGO resulted in remarkable stability for up to 90 days. Moreover, the 6-SRGO-LLPS demonstrated higher *in*

vitro cytotoxic activity in human breast cancerMCF-7 cells and *in vivo* anticancer activity in DAL cells than 6-SRGO, which may bring about reduction in dose as well as cost. Thus 6-SRGO-LLPS may be used to add value to new products by increasing anticancer activity, especially in functional foods directed to the breast cancer population, in which 6-SRGO play an important role on the attenuation of complications associated to cancer.

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CONFLICT OF INTEREST

The authors report no declarations of interest.

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