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Original Article

6-SHOGAOL RICH GINGER OLEORESIN LOADED MIXED MICELLES ENHANCES *IN VITRO* CYTOTOXICITY ON MCF-7 CELLS AND *IN VIVO* ANTICANCER ACTIVITY AGAINST DAL CELLS

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ABSTRACT

Objective: Ginger oleoresin (GO) plays an important role on the attenuation of complications associated to the cancer 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.

Methods: Thus in the present study, 6-SGL rich GO (6-SRGO) was incorporated into mixed micelles using phospholipid (Soya Lecithin) as a carrier. The prepared 6-SRGO loaded mixed micelles (6-SRGO-LMM) were characterized physically and chemically using Fourier transform infrared spectroscopy (FTIR), Differential scanning calorimetry (DSC) and further evaluated for stability study, *in vitro* release study, *in vitro* cytotoxicity study and *in vivo* anticancer activity in comparison with 6-SRGO.

Results: The composition such as, drug content (86.27±1.56), encapsulation efficiency (81.55±1.05) and particle size (356.11±4.07) were optimized using 3^2 factorial design. FTIR and DSC study confirm that the 6-SGL from 6-SRGO was entrapped in the core of phospholipid by self-assembly method to form mixed micelles. The 6-SRGO-LMM exhibited significant *in vitro* (GI50-23.2 µg/ml) and *in vivo* anticancer activity in comparison with 6-SRGO.

Conclusion: We have developed and investigated mixed micelles composed of phospholipids (soya lecithin S80) and SCH as an effective nanocarrier for the delivery of a natural lipophilic anticancer bioactive 6-SGL from 6-SRGO.

Keywords: 6-shogaol, Mixed micelles, Factorial designs, Breast cancer

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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 healthcare 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 [1, 2]. However, the low aqueous solubility, poor bioavailability, and the stability of phytoconstituents lead to hamper their use in the treatment of various diseases. Therefore, the various delivery system including nanotechnological-based drug-delivery systems has been designed for different naturally occuring phytoconstituents to increase the aqueous solubility and bio-availability and reduce the dose without hampering the efficacy of the drug, safety and the compatibility with patients [3].

6-shogaol rich ginger oleoresin (6-SRGO) is an oleoresin isolated from *Zingiber officinale* Rosc., consisting of higher content of phenolic ketones; gingerols and shogaols [4-6]. Traditionally they are used for the treatment of a cough, stomach upset, inflammation, hair diseases [7], diarrhoea, nausea and vomiting. Among the various phenolic constituents of ginger oleoresin (GO), the analogs of shogaols and gingerols have been known for their potent antioxidant, anti-inflammatory [8], antimicrobial [9] and anticancer activities [10]. Various studies have discovered that 6-shogaol (6-SGL) possesses most potent anticancer activity than the other analogs of shogaols and gingerols [11]. Various *in vitro* studies have been demonstrated the role of 6-SGL in suppressing the different cancer cells like ovary [12], lungs, colon, gastrointestinal tract and neuroblastoma. The metabolites of 6-SGL are also known to possess anticancer activity [2, 13].

The major challenge of using 6-SGL is its poor aqueous solubility; which limits its therapeutic efficacy [10-14]. Besides the 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 a conventional extract of ginger but detail investigation, characterization and anticancer potential are not reported [15-19]. To overcome these problems present research aims at incorporating 6-SRGO within polymers to develop a drug delivery system displaying enhanced drug solubility, while reducing systemic toxicity of 6-SRGO.

Mixed micelles; a system for the solubilisation of water-insoluble drugs to increase its efficacy [20, 21] is known for its stability and its ability to solubilize maximum amount of water-insoluble drugs in their inner core [22]. Mixed micelles are smaller in size and having outer hydrophilic shell which is responsible for prolonged circulation times *in vivo* and results in accumulation in the tumoral tissues. Mixed micelles also play an important role in pharmacokinetic and biodistribution behaviour of the drugs [23]. In addition, mixed micelles of 10–400 nm are advantageous for passive targeting into solid tumors by virtue of the enhanced permeability and retention (EPR) effect [24]. Such systems have been applied for delivery of various anticancer drugs such as doxorubicin, propofol, docetaxel and gambogic acid [25-29].

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

MATERIALS AND METHODS

Materials

Mixed micelles were produced using a carrier Phospholipid that was supplied by Cologne, Germany, with a melting point at 100 °C. The reference standard (pure isolated) of 6-shogaol (>96% purity w/w)

was purchased from Natural Remedies Pvt. Ltd. Bangalore, Karnataka, India. 6-SRGO was purchased from Nisarg Biotech, Satara, Maharashtra, India, which is totally free from any endotoxins. Dialysis bags (MW cut-off 12,000) were purchased from Sigma-Aldrich Chemical Private Ltd (Bangalore, India). The needed chemicals and reagents (analytical grade) used in this research work are purchased from Merck Specialties Private Limited, Mumbai, India.

Quantification of 6-SGL present in GO

6-shogaol present in ginger oleoresin was quantified using an RP-HPLC method which was reported in our earlier study [30].

Preparation of 6-SRGO-LMM

6-SRGO-LMM was prepared according to self-assembly method [31]. Extraction of 6-SGL was performed by adding the GO to a dehydrated ethanol containing a specific amount of phospholipid followed by mixing with a suitable quantity of sodium cholate hydrated (SCH) under stirring at room temperature. Sufficient distilled water was added under magnetic stirring at 2000 rpm for 45 min followed by sonication for 10 min to obtain 10 ml of the mixed micellar suspension. Blank mixed micelles (devoid of 6-SRGO) were prepared in a similar manner.

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

3^2 factorial designs were adopted to optimize the micelle composition while studying the effect of phospholipid and SCH (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-LMM

Micelles size distribution and zeta potential

The laser diffraction technique was used to determine the size of blank and 6-SRGO-LMM (Malvern 2000 SM; Malvern Instruments, Malvern, UK). The laser Doppler electrophoretic mobility measurements (Zetasizer 3000, Malvern Instruments) were done to measure the zeta potential at 25 °C.

DC and EE

The concentration of 6-SGL in the mixed micelles was determined by rapid and sensitive RP-HPLC method. The superficial 6-SGL was determined by washing 6-SRGO-LMM 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 6-SGL. The DC was determined as the ratio between the micelles collected at the end of the process and the mass of carrier added to 6-SRGO used in the preparation of micelles.

Physical characterization

Fourier transform infrared spectroscopy (FTIR)

The pure 6-SRGO and 6-SRGO-LMM were characterized by FTIR spectroscopy in the 4000 to 400 cm^{-1} region using FTIR Spectro-photometer (Jasco 4100).

Differential scanning calorimetry (DSC)

DSC was done to perform the thermal analysis of 6-SRGO and 6-SRGO-LMM by using DSC 821, Mettler Toledo instrument. Near about 5 mg of 6-SRGO-LMM were heated in a hermetically sealed aluminium pan with heating rate 10 °C per min under a nitrogen atmosphere (flow rate 50 ml/min). 6-SRGO was also analyzed for comparison.

Surface morphology

The morphology of 6-SRGO-LMM was performed using transmission electron microscopy (TEM). The sample preparation for the TEM was performed following the method reported in [3].

Evaluation of 6-SRGO-LMM stability

6-SRGO-LMM were transferred in glass containers and stored at 25 °C, protected from the light for 90 d to evaluate short-term stability studies. Micelles were evaluated with respect to the amount of 6-SGL, PS and EE.

***In vitro* release of 6-SRGO-LMM**

The *in vitro* release of 6-SRGO-LMM was carried out in 0.1N HCL (pH 1.2) and in phosphate-buffer (pH 7.4) using dialysis bag diffusion technique [3]. 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 incorporated into a dialysis bag (cellulose membrane, MW cut-off 12,000 Da), sealed and incorporated into 50 ml of release medium. The temperature of the entire system was maintained at 37±0.5 °C with constant magnetic stirring at 100 rpm/min. 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 mixed micelles and 6-SRGO-LMM 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 the method described by [3].

Acute toxicity study

The acute toxicity study was performed for 6-SRGO and 6-SRGO-LMM 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-LMM 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 behavioural changes, a 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 d for behavioural change and sign of toxicity.

***In vivo* anticancer activity of 6-SRGO and 6-SRGO-LMM against Dalton's Ascitic Lymphoma (DAL) in mice**

Male Swiss albino mice having the weight range of 20 gm to 25 gm were used for the study. They were kept in to micro nylon boxes at control temperature (temp 25±2 °C). The experimental protocol was approved by the 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 (Approved protocol no-CPCSEA/Q. A./01/2014-15; institutional approval no-CPCSEA/ 1999/100).

Induction of cancer cells

DLA cells were procured from Amala cancer research center, Trissur, Kerala, India. The cancer cells were implanted *in vivo* in mice by intraperitoneal route. Before transferring the cancerous cells to mice, by using saline solution the DLA cells were taken from the peritoneal cavity of the mice. The total cells count was maintained up to 1 x 10^6 after dilution and was given intraperitoneally. The treatment was started after seven days of induction of tumor cells [32, 33].

Grouping of the mice and outline of the treatment

Animals were grouped into nine groups with each group contains six animals. The animals of eight groups (group 2-8) received DLA cells (1 x 10^6 cells per mice), and group 1 was kept as a control group.

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

Group 3 represents positive control, (Received 5-fluorouracil injection at 20 mg/kg body weight, orally).

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

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

Evaluation of clinical parameters

During this study, the cancer cells were inoculated into the animals and the treatment for that cancer was started after 24 h one time a day for 14 d. Once after all the doses were finished, the 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 the content of Hb, RBC count, WBC count, platelet count and the volume of packed cells. Serum enzyme and the lipid profile include a count of total cholesterol (TC), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) and the derived parameters such as total lifespan (%) 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 Dunnetts post-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

We have developed and investigated mixed micelles composed of phospholipids (soyalecithin S80) and SCH as an effective nanocarrier for the delivery of a natural lipophilic anticancer bioactive 6-SGL from 6-SRGO to enhance both *in vitro* cytotoxicity and *in vivo* anticancer activity of 6-SGL.

Quantification of 6-SGL present in GO

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 a retention time of 10.30 min at the wavelength 281 nm (fig. 1). The amount of 6-SGL present in 6-SRGO was found to be 20%±2% whereas the optimized formulation 6-SRGO-LMM was found to have 16%±1% when compare to the pure isolated 6-SGL.

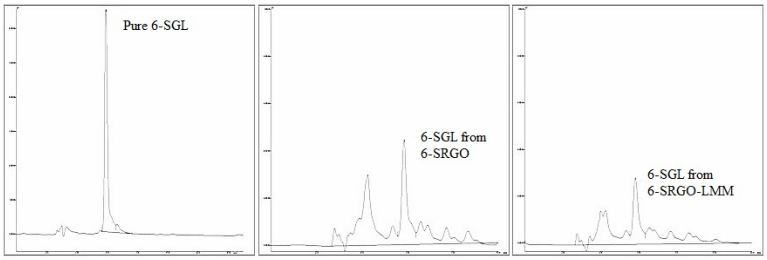

Fig. 1: HPLC chromatograms of pure 6-SGL (A), 6-SGL from 6SRGO (B) and 6-SGL from 6-SRGO-LMM (C) formulation

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

6-SRGO-LMM was produced and the effect of these prepared mixed micelles composition on DC, EE and PS was studied by 3^2 factorial designs [34]. The mean PS was observed in between 300–400 nm and was affected by the selected variables (r2 = 0.9247).

The DC and EE were in the range of 78–97% and 70–89% respectively. A good fit (r2 for DC = 0.7656 and EE = 0.8875) was observed between the DC, EE and the independent variables.

Fourier transform infrared spectroscopy

In the IR spectra of 6-SRGO (fig. 2A), following peaks are assigned,-OH stretching (~3124 cm^{-1}), Aromatic –CH stretching (~2994 cm^{-1}), Aliphatic–CH stretching (~2852 cm^{-1}), -C=O stretching (~1736 cm^{-1}) and OH bending out of the plan (~1373 cm^{-1}), while in 6-SRGO-LMM (fig. 2C), 6-SRGO incorporated with polymer phospholipid, the remarkable peak of an active drug such as–C=O ketone stretching and–OH bending were not seen.

Differential scanning calorimetry

6-SRGO-LMM and 6-SRGO were analyzed by Differential scanning calorimetry to determine the onset, maximum temperature and enthalpy (table 1 and fig. 3).

Table 1: Differential scanning calorimetry analysis

	A	B
Peak (°C)	240.65	148.26
Onset (°C)	223.87	135.51
Enthalpy (J/g)	31.11	56.74

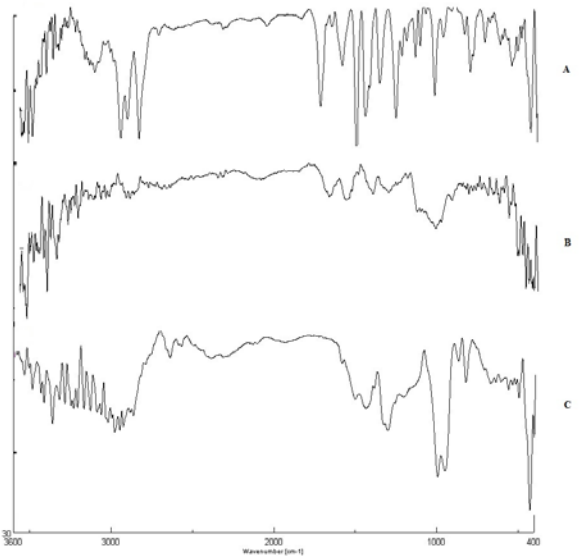

Fig. 2: Fourier transform infrared (FT/IR) spectroscopy analyses of (C) 6-SRGO-LMM, compared with (A) 6-SRGO and (B) Phospholipid

Surface morphology

The obtained 6-SRGO-LMM solution appeared clear and light yellowish in color (fig. 4). The fig. also shows the suspension of pure 6-SRGO dispersed in water at the same drug concentration similar to that reported in the literature [35].

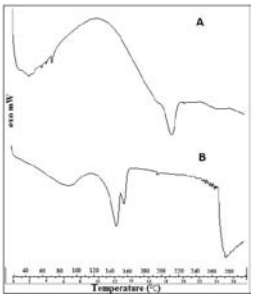

Fig. 3: Differential scanning calorimetry (DSC) analyses of (A) 6-SRGO and (B) 6-SRGO-LMM

Fig. revealed that the prepared formulation was monodispersed, tightly packed and spherical shaped mixed micelles whose size measured by laser scattering technique was correlated well with that by TEM.

Evaluation of 6-SRGO-LMM stability

Stability study reveals that 6-SRGO-LMM did not show noticeable changes in their size (340±15 nm) and EE (89±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.

This indicated that the drug-loaded micelles were physically stable at room temperature for at least 3 mo.

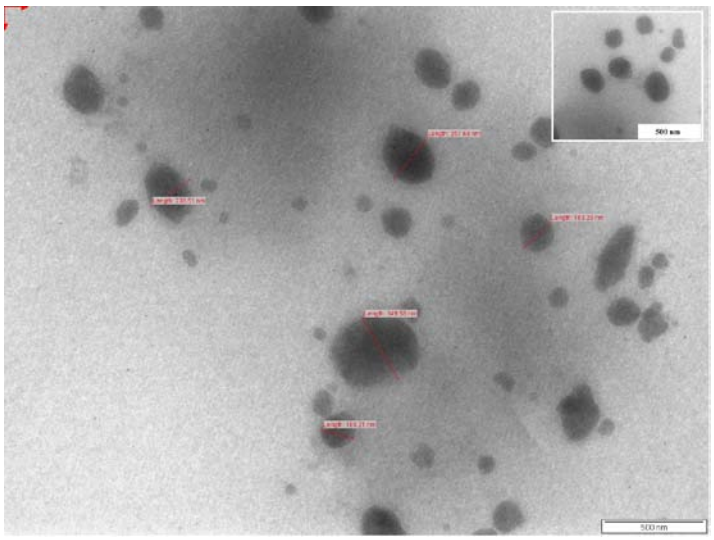

Fig. 4: Transmission electron micrograph of optimized 6-SRGO-LMM

***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-SGL from 6-SRGO-LMM in comparison with 6-SGL from 6-SRGO was investigated in gastric fluids (pH 1.2), and intestinal fluids (pH 7.4). Fig. 5 showed the dissolution profile of 6-SGL from 6-SRGO-LMM and 6-SRGO in both HCl (pH 1.2) and phosphate buffer saline (pH 7.4), respectively. 6-SGL release from the mixed micellar system was always slower than the corresponding 6-SGL from 6-SRGO 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-LMM showed a very slow release of 6-SGL approximately 30% within 2 h and the release was over 95 % by 42 h.

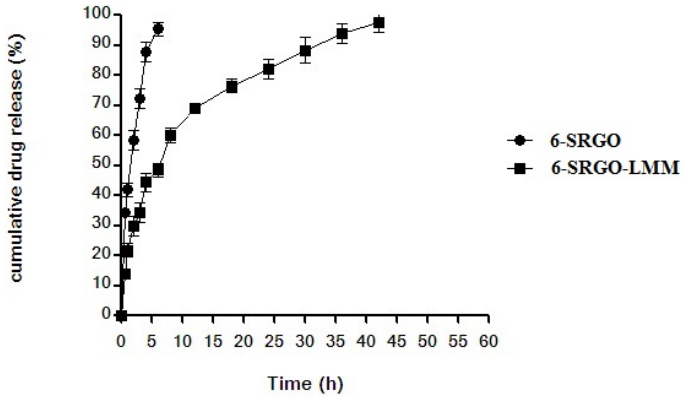

Fig. 5: *In vitro* release profiles of 6-SGL from 6-SRGO and from 6-SRGO-LMM

***In vitro* anticancer activity**

The *in vitro* anticancer activity of 6SRGO-LMM was investigated and compared with the free drug in solution, blank mixed micelles and marketed Adriamycin (Doxorubicin) against human breast cancer MCF-7 cells using *in vitro* SRB assay. The results illustrated in table 2, indicated that 6SRGO-LMM displayed better cytotoxic activity than the 6-SRGO and the blank mixed micelles. The total growth

inhibition concentration against MCF-7 was found to be 23.2 µg/ml, 26.8 µg/ml and>80 µg/ml for 6-SRGO-LMM, 6-SRGO and blank respectively. LC50 against MCF-7 of 6-SRGO-LMM was>80 µg/ml, whereas free 6-SGL from GO in solution and blank mixed micelles showed>80 µg/ml. The representative images are shown in fig. 6.

Table 2: *In vitro* cytotoxic effect of 6-SRGO-LMM on MCF-7 breast cancer cells

	Drug concentration (µg/ml) calculated from graph		
MCF 7	**LC50**	**TGI**	**GI50**
6-SRGO	>80	56.4	26.8
Blank	>80	>80	>80
6-SRGO-LMM	>80	55.9	23.2
ADR	60.8	29.5	<10

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

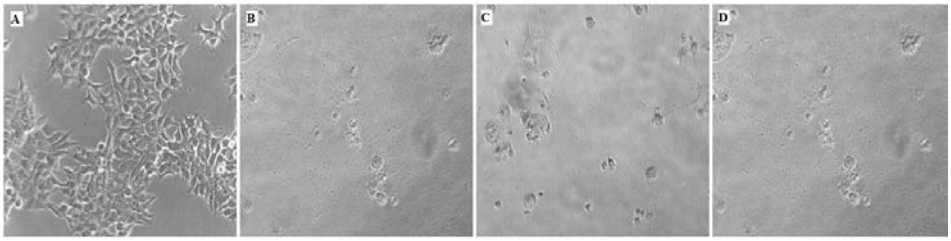

Fig. 6: *In vitro* cytotoxicity study on the breast cancer cell line (MCF 7), (A) MCF 7 control, (B) Treatment control, (C) Treated with 6-SRGO, (D) treated with 6-SRGO-LMM

Acute toxicity study

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

***In vivo* anticancer activity of 6-SRGO and 6-SRGO-LMM against dalton's ascitic lymphoma (DAL) in mice**

In the *in vivo* anticancer activity, the average lifespan of tumor control group of animals was found to be 48% whereas 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 88.5%, 89.5% and 90.5% for group treated with 6-SRGO-LMM 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 the very potent cytotoxic nature of 5-FU [36].

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

Table 3: Effect of 6-SRGO and 6-SRGO-LMM on hematological parameters

Treatment	Total WBC cells/mlx10^3	Rbc count mill/cumm	Hb gm/dl	PCV %	Platelets 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	12.34±1.55^{b**}	4.06±1.72^{b**}	12.24±1.55^{b**}	17.38±2.40^{b**}	3.26±0.84^{b**}
G_8	12.22±1.50^{b**}	4.10±1.75^{b**}	12.28±1.58^{b**}	17.30±2.35^{b**}	3.30±0.86^{b**}
G_9	11.80±1.42^{b**}	4.15±1.78^{b**}	12.35±1.62^{b**}	17.26±2.30^{b**}	3.35±0.88^{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-LMM 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 Dunnetts post 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 induction of DLA cells, rises the level of cholesterol, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase to the significant level when compared to the normal group. The treatment with 6-SRGO-LMM recovers the above mentioned changes to the normal level than the groups treated with 6-SRGO at all three doses (table 3).

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

DISCUSSION

6-SGL from ginger has been well established for its anticancer potential. However, its usage as a therapeutic anticancer agent is limited due to its poor water solubility and bioavailability. As per the best knowledge of authors, 6-SGL in pure form is reported to be unstable in simulated gastric and intestinal fluids in GI [37], thus expected to be unstable when administered orally. At the best of our knowledge, there have been no studies reported to formulate 6-SGL to solve the above problems. Obtaining pure compound from herbals by tedious isolation processes and formulating them are quite expensive,

moreover, single phytocompounds in most cases exhibit less pharmacological activities than when administer in the form of enriched extracts due to synergistic activity. Thus in present study, an attempt has been made to formulate the 6-SRGO-LMM composed of phospholipid (soya lecithin S80) and SCH as an effective nanocarrier for the delivery of a natural lipophilic anticancer bioactive 6-SGL.

Table 4: Effect of 6-SRGO and 6-SRGO-LMM on serum enzymes and lipid proteins

Treatment	Cholesterol (mg/dl)	TGL (mg/dl)	AST (U/l)	ALT (U/l)	ALP (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	118.30±3.50^{b**}	164.25±2.72^{b**}	43.32±2.34^{b**}	35.32±1.58^{b**}	164.20±2.30^{b**}
G_8	116.44±3.46^{b**}	163.32±2.70^{b**}	42.35±2.34^{b**}	34.92±1.52^{b**}	162.16±2.22^{b**}
G_9	114.52±3.40^{b**}	158.30±2.48^{b**}	41.95±2.22^{b**}	34.60±1.46^{b**}	160.22±2.18^{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-LMM 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 Dunnetts post 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

Table 5: Effect of 6-SRGO and 6-SRGO-LMM 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 X 10^6
G_1	6	>>30 d	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	88.5%	5.60±0.75^{b**}	1.44±0.32^{b**}
G_8	6	89.5%	5.58±0.70^{b**}	1.40±0.26^{b**}
G_9	6	90.5%	5.55±0.64^{b**}	1.37±0.23^{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-LMM 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 Dunnetts post 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

A series of 6-SRGO-LMM were prepared and observed physically for phase separation. In the process of reformulations study, the concentrations of phospholipid and SCH that would give non-coagulation and non-sedimenting mixed micelles were determined. A 3^2 factorial design was taken to optimize their concentrations. The concentration of 6-SRGO was kept constant. As per 3^2factorial designs, nine different batches of the different concentration ratio of phospholipid and SCH were prepared. The responses obtained from these batches are shown in table 6. The data obtained was subjected to multiple regression analysis using design expert 10 software.

Table 6: Regression analysis results of measured responses

Coefficients	Parameters		
	Micelles size	Drug content	Encapsulation efficiency
β_0	356.11	86.27	81.55
β_1	50.33	-0.47	6.22
β_2	12.67	4.82	1.61
β_{11}	-	-	-
β_{22}	-	-	-
β_{12}	-	-	-3.60
r^2	0.9247	0.7656	0.8875

The results of multiple regression analysis of the obtained data are summarized in table 6. 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 (fig. 7).

From the factorial design study (table 6), it is observed that positive coefficients of the main term X1 and the interaction term X1X1 indicated a favorable effect on the mean micelle size, with the phospholipid chiefly influencing than SCH. The phospholipid had a linear effect on the mean micelles size as seen in surface plot (fig. 7A). Smaller micelles were obtained at low phospholipid content, probably due to high distribution efficiency of an internal phase into external phase [3]. Increase in the viscosity of internal phase with an increased amount of phospholipid also provides resistance for mass transfer during diffusion of an internal phase into the external phase leading to micelles enlargement. The increase in the mean micelles size may also be due to the hydrophobic-hydrophobic interaction of phospholipid with the drug.

Both X1 and X2 showed favorable positive effects on DC and EE. In case of phospholipid, it showed the linear effect on DC and EE.

Further more in case of SCH it also showed linear effects on DC and EE. As seen in surface plot (fig. 7B, C), the phospholipid favored DC and EE by hydrophobic-hydrophobic interactions with drugs leading to the formation of interpenetrated network chain. However, SCH exerted an opposite effect as it led to solubilization of the drug in the external phase.

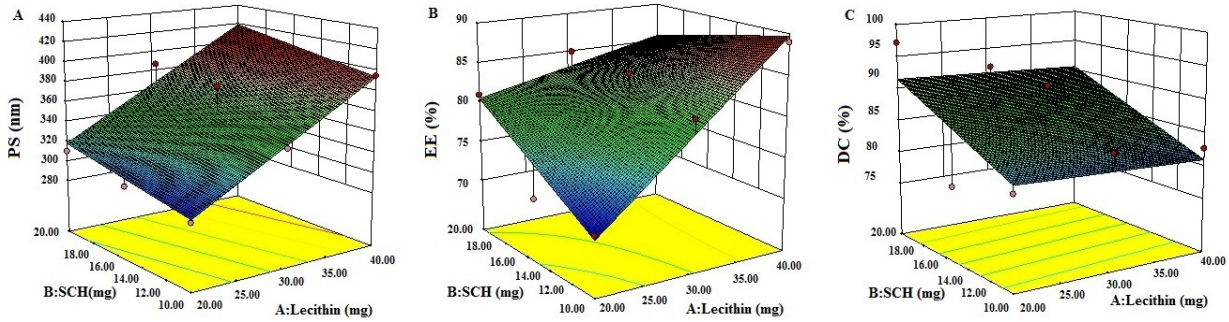

Fig. 7: Response surface plot illustrating effect of factorial variables. (A) Particle size, (B) Encapsulation efficiency, (C) drug content

Based on the results of the factorial design, batch F5 6-SRGO-LMM having acceptable PS, DC and EE was selected as an optimized batch. The size of the optimized 6-SRGO-LMM was 380±3 nm–which was not significantly different from its blank counterpart. 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) [24]. Drug delivery to specific sites of the body is influenced by the size of the mixed micelles; smaller micelles may tend to minimize the particle uptake by nontargeted cells, including their premature clearance by the mononuclear phagocytic system [38]. It is hypothesized that mixed micelles developed in the present study are of appropriate size to be able to passively target the tumor site.

In the IR spectra of 6-SRGO-LMM does not shows a remarkable peak of an active drug such as–C=O ketone stretching and–OH bending, thus final IR spectra of 6-SRGO-LMM revealed that 6-SRGO has been completely encapsulated with phospholipid in the formulation.

According to the DSC results, the melting point of free 6-SRGO was observed at around 240 °C with the enthalpy 31.11 J/g. In case of 6-SRGO-LMM thermogram, the free 6-SRGO peak was disappeared and shifted to 148 °C indicating molecular dispersion of 6-SRGO inside mixed micelles. Thermograms of 6-SRGO-LMM show a sharp endothermic peak starting near about at 135 °C and ending at 155 °C. Onset temperature at approximately 135 °C and melting point around 148.26 °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 [39].

The TEM image shows that there is no drug crystallization appears in the micellar formulations and the surface of the micelles are smooth and they are suspended uniformly in the formulation. The bright region may represent the hydrophilic shell, while the dark region was likely the hydrophobic core of the micelles.

The enhanced *in vitro* cytotoxic activity of 6-SRGO-LMM may be attributed to greater cellular uptake of micelles via phagocytosis or the fusion process of phospholipid micelles as compare to 6-SRGO [40]. Therefore, 6-SRGO-LMM might have served as a potential nanocarrier to improve the *in vitro* cytotoxic activity of 6-SGL. The lower anticancer activity of 6-SRGO and blank mixed micelles may be due to their efflux by P-glycoprotein pumps. The above GI50 and LC50 values reveal that the 6-SRGO-LMM gives potential anticancer activity as compared to 6-SRGO.

Acute toxicity study showed that oral administration of 6-SRGO and 6-SRGO-LMM 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 [41]. 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.

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 an ascitic fluid with tumor growth to meet the nutritional requirement of tumor cells [36]. Treatment with 6-SRGO-LMM increased the life span of the mice, controlled the tumor volume and reduced the cell count of the tumor more significantly than the groups treated with 6-SRGO at a dose of at all three doses. The lifespan 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 [42].

Usually, the major problem in the treatment of the cancer is the suppression of myeloma and the anemia [43, 44]. The anemia arises mainly because of reduction in the RBC count or reduction in the total hemoglobin count [45]. Treatment with 6-SRGO and 6-SRGO-LMM at all three doses brought backs all the hematological parameters to the normal level significantly.

Thus the results show that the 6-SRGO-LMM exhibited better antitumor activity against DLA bearing mice as compare to the 6-SRGO at all three doses.

CONCLUSION

We have developed and investigated mixed micelles composed of phospholipids (soya lecithin S80) and SCH as an effective nanocarrier for the delivery of a natural lipophilic anticancer bioactive 6-SGL from 6-SRGO. The developed 6-SRGO-LMM exhibited higher DC and sustained release of 6-SGL from 6-SRGO. Micellar encapsulation of 6-SRGO resulted in remarkable stability for up to 90 d. Moreover, the 6-SRGO-LMM demonstrated higher *in vitro* cytotoxic activity in human breast cancer MCF-7 cells and *in vivo* anticancer activity in DAL cells than 6-SRGO, which may bring about reduction in dose as well as cost. As a result, this 6-SRGO-LMM may be used to add value to new products by increasing anticancer activity, in which 6-SRGO play an important role on the attenuation of complications associated to cancer.

ABBREVIATION

GO-ginger oleoresin, 6-SGL-6-shogaol, 6-SRGO-6-shogaol rich ginger oleoresin, 6-SRGO-LMM-6-shogaol rich ginger oleoresin-loaded mixed micelles, FTIR-fourier transform infrared spectroscopy, DSC-differential scanning calorimetry, SCH-sodium cholate hydrated, EPR-enhanced permeability and retention, LMM-loaded mixed micelles, RP-HPLC-reverse phase high performance liquid chromatography, DC-drug content, EE-entrapment efficiency, PS-particle size, TEM-transmission electron microscopy, ACTREC-advanced centre for treatment, research and education in cancer, CPCSEA-committee for the purpose of control and supervision on experimental animals, DAL-dalton's ascitic lymphoma, IAEC-institutional animal ethics committee.

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AUTHOR CONTRIBUTION

Kiran Kemkar: Literature survey, data collection and analysis, practical work, drafting the article, writing manuscript.

Dr. Sathiyanarayanan L.: Design of the work, data analysis, critical revision of the article, final approval of the version to be published.

Dr. Arulmozhi Sathiyanarayanan: Design and analysis of animal activity.

Dr. Kakasaheb Mahadik: Final approval of this work and help to get the facility available for the work to be done.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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