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Preparation, Characterization, and *In Vitro* Release Study of Resveratrol-Laminarin-Loaded Liposomal Formulation

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Abstract

The present study aims to formulate and evaluate the physicochemical characteristics of the resveratrol-laminarin encapsulated liposomal formulation. Resveratrol is a phytoalexin with numerous biological effects like antidiabetic, anti-oxidant, anti-cancer, and anti-inflammatory properties. A polysaccharide extracted from brown algae called laminarin possesses anti-tumor, anti-oxidant, anti-coagulant, anti-inflammatory, and antiobesity properties. Resveratrol, a hydrophobic drug, is entrapped in the liposomal bilayer, and laminarin, a hydrophilic drug, is enclosed in the hydrophilic core. The preparation and characteristics of resveratrol-laminarin-loaded liposomes, including their particle size, zeta potential, entrapment efficiency, and in-vitro release kinetics, were investigated in this study. The thin film lipid hydration method was used to prepare the liposome using soy phospholipids and cholesterol in a 6:1 ratio. Downsizing of the prepared liposomes was carried out by homogenization and sonication techniques. The liposome had a particle size of 120.7 nm after sonication, and a zeta potential of -33.4 mV was observed. The highest entrapment efficiency recorded for resveratrol is 90.69%, and 83.64% for laminarin. Biophysical characterisation of the prepared liposomes was carried out by scanning electron microscope and UV-visible spectrophotometry. The release study of resveratrol and laminarin was observed for six hours. A stable and steady release of the compounds was observed. The stability of the liposomes stored at 4ºC was analyzed after three months of preparation. In conclusion, the prepared liposomal formulations exhibited good physicochemical characteristics and are of great therapeutic value in the future.

Keywords: resveratrol, laminarin, liposome, *in vitro* release, encapsulation efficiency

Introduction

Liposomes are artificially made spherical vesicles containing phospholipids and cholesterol (1). They are classified based on size and bilayer into multilamellar vesicles, unilamellar vesicles, large unilamellar, and small unilamellar vesicles, which are used to deliver hydrophobic and hydrophilic drugs (2). Downsizing of the prepared large unilamellar vesicles is achieved by methods like extrusion, homogenization, and sonication (3,4). Liposomes are suitable drug carriers as they are biodegradable, biocompatible, and have low toxicity (5). Resveratrol has many therapeutic effects, like antiobesity, antidiabetic, antioxidant, and anti-inflammatory properties, and is found primarily in

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red wine, peanuts, and berries (6). Resveratrol has proven anti-thrombotic and anti-inflammatory effects making it a promising treatment option for COVID-19 (7). Resveratrol has a potent anti-adipogenic impact by preventing fat storage processes and igniting the oxidative and lipolytic pathways, demonstrating their cardioprotective properties by preventing platelet aggregation (8). The strong anti-inflammatory property of resveratrol also helps in combating inflammatory bowel disease (9). Laminarin is a polysaccharide isolated from brown seaweed and has anti-oxidant, anticoagulant, anti-tumor, and anti-inflammatory properties (10). Laminarin has potent osteogenesis and angiogenesis properties which help in bone regeneration (11). Laminarin also inhibits α-glucosidase and α-amylase, proving to be a good antidiabetic agent (12). Both compounds, when used together, can target diseases like diabetes, obesity, and cancer and also reduce the comorbidities associated with the disease, which reduces the cost and side effects of multiple drug usage for a single disease (13). Resveratrol and laminarin, being phytochemicals, are prone to easy degradation in stomach acids and have lesser bioavailability (14). This gives rise to the need for new techniques for their delivery. These challenges are overcome by liposomal encapsulation of the compounds, which also ensures a regulated release of the encapsulated compounds to the target site (15). Resveratrol, being hydrophobic, gets encapsulated in the bilayer membrane, and hydrophilic laminarin gets encapsulated in the hydrophilic core of the liposome, which provides a clear separation of the compounds from each other (16). The encapsulation of resveratrol and laminarin into a single liposome has not been performed to date, which marks the importance of characterization of the prepared liposomes and optimization of the drug release activity.

The present study intended to encapsulate resveratrol and laminarin into a liposomal formulation. Physical morphology, particle size, zeta potential, encapsulation efficiency, and in-vitro release of the produced liposome were all performed.

Materials and Methods

Resveratrol and Laminarin were purchased from Sigma Co. Ltd (America), and Soybean phosphatidylcholine and Cholesterol were purchased from Himedia Laboratories Ltd (Mumbai, India). Analytical grade reagents and solvents were used throughout the experiment.

Preparation of resveratrol laminarin-loaded liposomes

Liposome encapsulated resveratrol-laminarin formulation was prepared by theconventional thin film lipid hydration method (17) with slight modifications. Briefly, soy-phosphatidylcholine and cholesterol were combined in a 6:1 ratio and dissolved in chloroform that contained 1% methanol. Resveratrol was dissolved in chloroform containing 1% methanol, and laminarin was dissolved in distilled water. Contents were mixed, and rotary evaporation (Rotary evaporator Superfit, PBU6) was performed at 55ºC and 50 rpm under controlled pressure to remove the organic solvents. Using phosphate buffer saline with a pH of 7.4, the thin film was rehydrated and left overnight at 4 ºC. Downsizing of the liposomal formulation was achieved by homogenization (Homogenizer REMI, RQ127A/D) at 5000 rpm and ultrasonication (Sonicator Sonics, VCX130) at 60% amplitude and 10s pulse interval for 1 hour (18).

Free drug separation

Using a cooling centrifuge (Cooling centrifuge Lark LIMR96) at 20,000 g for 1 hour at 4ºC, the free unentrapped drugs were separated from liposomes containing resveratrol and laminarin. The supernatant was discarded, and the resuspended pellet was used for further studies.

Determination of drug entrapment efficiency

Liposomes were lysed using methanol and sonication for 10 minutes. The total resveratrol encapsulated was evaluated using the Folin-Ciocalteu reagent method (19), and the total laminarin encapsulated was evaluated

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using the phenol-sulphuric acid method (20). A UV-visible spectrophotometer was used to measure the entrapment efficiency of the resveratrol and laminarin-encapsulated liposomes for four different concentrations. The encapsulation efficiency of the resveratrol-laminarin encapsulated liposomes was calculated using the following formula:

$$\text{Encapsulation efficiency \%} = \frac{\text{(Absorbance of control-Absorbance of sample)}}{\text{(Absorbance of control)}} \times 100$$

Liposome size and zeta potential measurement

2ml of liposomal formulations was diluted in distilled water for the analysis(21). Liposome size and zeta potential were measured using a nanoparticle analyzer Horiba scientific SZ-100 by dynamic light scattering. The size of liposomes is expressed as the average diameter (z-average) calculated from the signal intensity.

Scanning electron microscopy of resveratrol-laminarin-loaded liposomes

To prepare the samples for scanning electron microscopy, an equal volume of liposomal solution was diluted with 0.1M non-saline phosphate buffer. Following the protocol mentioned in Mfuh et al. (22) the diluted sample was treated with 30 µl solution of ammonium molybdate solution of 11 % (w/w; pH 7.2) and dried in the open air at room temperature in a coverslip. The slides were then rinsed with 70 % ethanol. The slides were then sputter-coated with gold/palladium, and images were obtained at a magnification of 10 µM using Carl Zeiss Evo/18 scanning electron microscope.

In vitro drug release studies

Following protocol Ahmad et al. (23) and with slight modifications, the drug released was determined using a dialysis membrane method. A dialysis membrane (12000 molecular weight cut off; Sigma-Aldrich) was soaked in PBS buffer for 20 minutes, and 1 ml of liposomal solution was pipetted into a dialysis bag, sealed the ends with clips, and placed in the beaker containing PBS buffer (7.4 pH) at 500 rpm in a magnetic stirrer at 37 ºC. Samples were evaluated for 6 hours, and at an interval of 1 hour, 1 ml was collected from the beaker and determined the drug release spectrophotometrically as drug release percentage using the formula,

$$\text{Drug release \%} = \frac{\text{(Absorbance of control-Absorbance of sample)}}{\text{(Absorbance of control)}} \times 100$$

Stability study

To evaluate the stability of resveratrol-laminarin encapsulated liposomes, the formulation was stored for 3 months at 4ºC, and particle size and zeta potential were determined as a function of storage time (24).

Results and Discussion

Resveratrol and laminarin were encapsulated as liposomal formulations using the thin film lipid hydration method. Non-toxic phosphatidylcholine was used in the preparation of liposomes. Cholesterol was used in a small quantity to increase the rigidity and stability of prepared liposomes. Sonication and homogenization were performed to obtain the desired size and prevent aggregation. The physicochemical and surface morphology of the prepared liposomes were studied.

Entrapment efficiency of resveratrol and laminarin

The drug entrapped within the liposomes during the preparation is termed entrapment efficiency (25). Unentrapped drugs from liposomes were removed by centrifugation. The entrapment efficiency for four different concentrations (25, 50, 100 and 200 µg/ml) of drugs carrying liposomal formulations were tested. The Folin-Ciocalteu method was used to determine the entrapment efficiency of resveratrol, and laminarin was determined using the phenol-sulphuric acid method. The highest entrapment efficiency of resveratrol was 90.69% and 83.64 % for laminarin. Entrapment efficiency gradually decreased as the concentration of the drug increased. The entrapment efficiencies for four different concentrations of drug-encapsu-

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lated liposomes are depicted in Fig. 1.

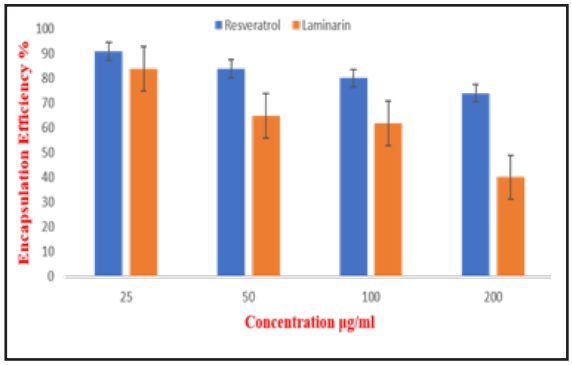

Fig. 1: Comparison of entrapment efficiency of encapsulated resveratrol and laminarin ranging from concentrations 25, 50, 100 and 200 µg/ml

Liposomal size, zeta potential, and surface morphology analysis

The size and the zeta potential of resveratrol-laminarin encapsulated liposomes were determined using a nanoparticle analyzer Horiba scientific SZ-100, and a size of 120.7 nm was obtained for the concentration with the highest entrapment efficiency depicted in Fig. 2. An ideal negative zeta potential value was recorded, which suggested that the liposomes were not aggregated (Fig. 3). The surface morphology of liposomes was determined using a scanning electron microscope. The liposomes obtained were spherical and not aggregated (Fig. 4). No disrupted liposomes were found.

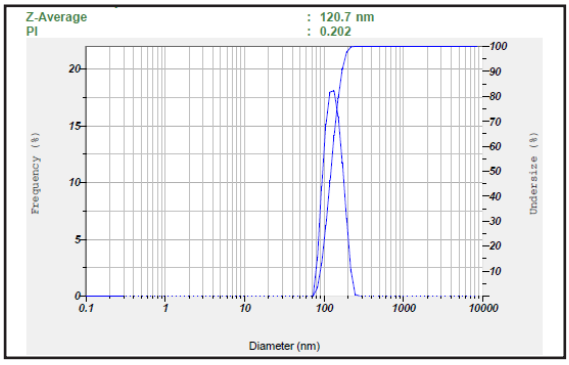

Fig. 2: Particle size distribution graph of resveratrol-laminarin encapsulated liposome

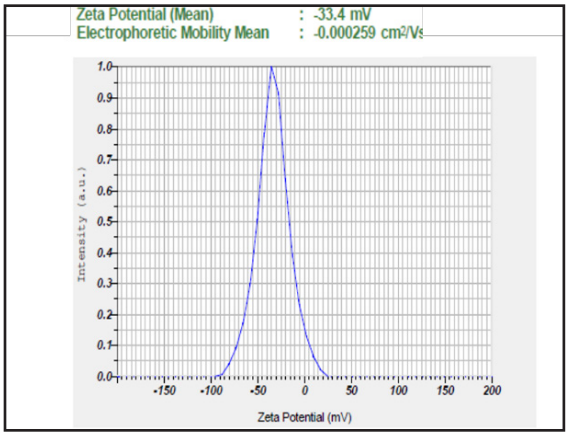

Fig. 3: Graph demonstrating the zeta potential value of resveratrol-laminarin encapsulated liposomes

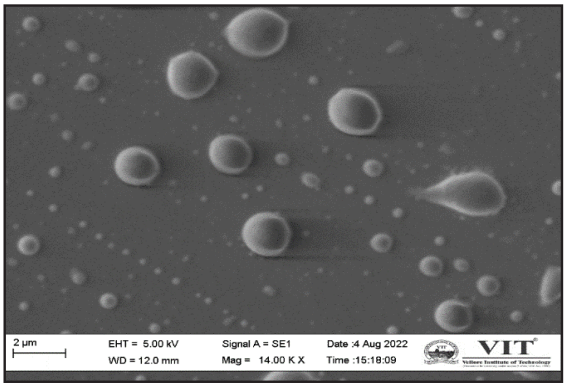

Fig. 4: Scanning electron microscope image of resveratrol-laminarin encapsulated liposomes

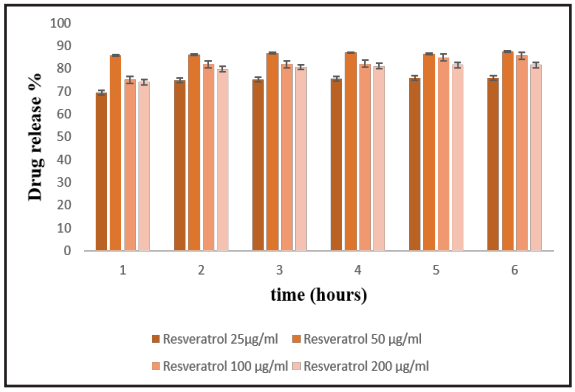

Fig. 5a: Comparison of in vitro release of resveratrol from liposomes ranging in concentrations of 25, 50, 100 and 200 µg/ml

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In vitro drug release studies

The release study of resveratrol and laminarin from the encapsulated liposomes was evaluated *in vitro* separately for four concentrations (25, 50, 100 and 200 µg/ml). Both drugs showed a slow and steady release. Phytochemicals have a very rapid release which accounts for their lesser bioavailability. On the contrary, the results obtained for liposomal encapsulated resveratrol and laminarin showed a steady release which shows a promising result for therapeutic use. Resveratrol showed the best release profile at 50µg/ml concentration and laminarin at 100µg/ml, as depicted in Fig. 5a and Fig. 5b. A liposomal formulation with these two drug concentrations can be optimized for further studies.

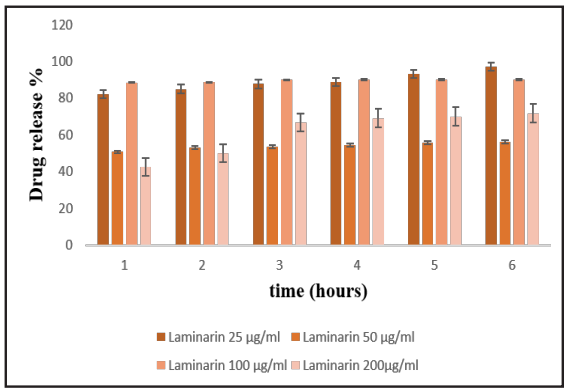

Fig. 5b**:** Comparison of in vitro release of laminarin from liposomes ranging in concentrations of 25, 50, 100 and 200 µg/ml

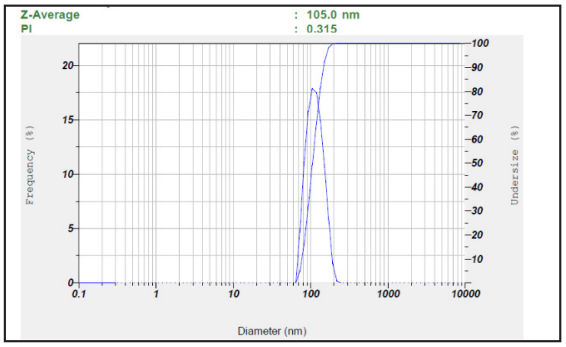

Fig. 6: Graph depicting the particle size of resveratrol-laminarin loaded liposome after storing for 3 months in 4ºC.

Stability study

The liposomal formulation was stored at 4ºC for three months. The particle size of the prepared liposomes and their zeta potential were evaluated to determine the stability of the stored liposomes. The particle size was 105 nm, and a negative zeta potential value was observed for the stored liposome (Fig. 6 and Fig. 7). The size reduction can be due to the shrinkage of liposomal vesicles over time, which explains the lesser aggregation due to the large negative charge on them (26).

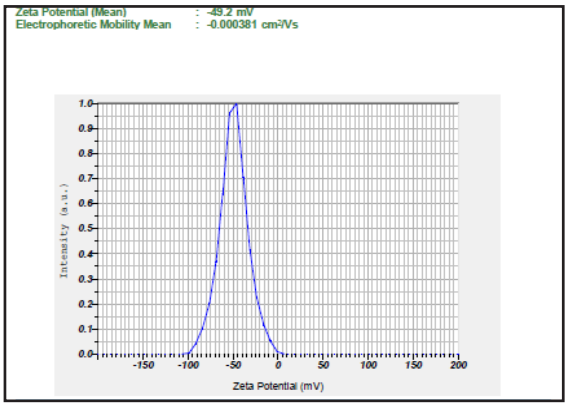

Fig. 7**:** Graph depicting the zeta potential of resveratrol-laminarin loaded liposome after storing for 3 months at 4ºC

Conclusion

Resveratrol and laminarin are phytochemicals with proven therapeutic properties. The lesser bioavailability, low stability, and poor pharmacokinetic activity of both drug account for the need for a better delivery system. Both drugs differ in their chemical properties, and it isn't easy to deliver them both together. To overcome this difficulty, resveratrol and laminarin can be encapsulated in the liposome, by which resveratrol will be entrapped in the lipid bilayer and laminarin in the hydrophilic core. Characterization of the prepared drug-encapsulated liposomes showed excellent results with reference to its particle size, zeta potential, and entrapment efficiency. The drug release study showed a stable and steady release which is an

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indication of a better half-life time and improved bioavailability. Further *in vitro* and *in vivo* studies pave a greater way for many therapeutic areas, including lifestyle diseases like diabetes and obesity.

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