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Opinion paper

Development of liposomal formulations of the eggplant glycoalkaloids solasonine and solamargine

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ABSTRACT

The eggplant glycoalkaloids solasonine and solamargine are efficient biomacromolecules against skin diseases but are water-insoluble which results in inefficient treatment due to inadequate transdermal delivery. To address this problem, several liposomal formulations were prepared and evaluated for parameters including lecithin type, hydration temperature, and pH. The optimal formula with high physical and chemical stability included the lecithin Phospholipon 80H hydrated with 10 mM NaCl (pH 5.5). Solasonine - solamargine loaded liposomes were tested for their physical and chemical stability and drug leakage over a three-month period. Furthermore, the drug release profile of the loaded liposomes was evaluated with different release media. The glycoalkaloids and their liposomal formulations were assessed for their biological activity in culture using HaCaT and SCC-25 cell lines. This work resulted in a biologically effective liposomal formulation that was stable (size <220 nm, PDI <0.25, encapsulation efficiency >80%) for at least three months.

1. Introduction

In the last few decades, nanotechnology has become a major player in many fields including pharmacology and molecular medicine, allowing advances in disease prevention, diagnosis, and treatment [1]. In addition, the examination of diseases at the molecular level has resulted in the discovery of new effective drug molecules. However, accurate targeting and delivery of these new drugs still pose significant obstacles for efficient, effective treatment. In recent years biomacromolecules have been replacing chemically-produced therapeutics as they offer more effective and less immunogenic treatments [2]. Although biomacromolecules have captured the attention of researchers due to their excellent in vitro therapeutic effects, the majority of these molecules are water-insoluble, which is the main cause of their poor bioavailability. Another problem with water-insoluble drugs is precipitation. These molecules tend to precipitate on varying organs and tissues, which is another cause of their poor bioavailability. The graver and more obvious danger of precipitation is the potential toxic effect of the drug molecule at the site of precipitation [3]. Nanocarrier systems can overcome the bioavailability issues of both lipophilic and hydrophilic molecules by improving their solubility [4]. Nanocarriers are not only helpful for enhanced cellular uptake of drugs but can also be used to increase drug accumulation at the disease site via various targeting strategies [5]. It was shown that administration of a therapeutic agent via a carrier has a 10-to-100-fold higher accumulation at the disease site compared with administration of the agent in its free form [6].

Lipid-based nanocarrier systems are biocompatible, renewable, non-toxic, biodegradable, self-targeting, and have longer blood circulation periods compared to synthetic carriers [7]. Liposomes, a type of lipid-based nanocarrier, consist of phospholipid bilayers and have been a focus of research due to their benefits which include increased bioavailability, improved cellular uptake, and stabilized therapeutics. An advantage of using amphiphilic phospholipids is that the liposomes can carry a wide range of both hydrophobic and hydrophilic drug molecules [8]. Also, since these drug molecules are entrapped within the vesicle, their off-target activity is eliminated. Transdermal skin drug delivery studies have focused on lipid-based drug carrier systems as their lipid composition is similar to those of the stratum corneum (SC), one of the two layers of the epidermis [9]. Treatment of skin-related diseases, like cancer and cutaneous and inflammatory disorders, is a major challenge because of the low diffusion rate of drug molecules through the SC which is the main barrier for transdermal drug delivery [10,11]. In addition, the rapidity of skin renewal (14 days) means that drugs are usually applied at high doses which creates problems with side effects.

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Melanoma, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC) are the main types of skin cancer. BCC and SCC are non-melanoma cancers that originate from keratinocytes. These types of cancers are more common, but melanoma is more lethal [12]. Among keratinocyte cancers, oral squamous cell carcinoma (OSCC) is a highly aggressive skin cancer that accounted for 350,000 new cases and 170,000 deaths in 2018 [13]. Treatment of this disease includes surgery, chemotherapy, radiotherapy, or a combination of these approaches. Because of the severe side effects of such treatments, the discovery of new anticancer drugs is crucial. Plants are the source of many compounds with various bioactivities. In this context, steroidal glycoalkaloids (SGAs), a well-known subgroup of alkaloids, are effective medicinal compounds and are produced mostly by the Solanaceae family [14]. As members of the Solanaceae, eggplant species produce the valuable steroidal glycoalkaloids solasonine and solamargine, which have many biological activities including anticancer activities. It has been shown that solasonine and solamargine are cytotoxic to many cancer cell lines including skin tumors [15]. To date, SS and SM have been tested on healthy and cancer cell lines of epithelial origin including VERO p35, MDA-MB-231, MCF-7, SH-SY5Y, SK-BR3, Eahy926, HepG2, SW480, and U87 [16–20]. In these studies, IC_{50} values for cancerogenic cell lines ranged from 3.96 μM to 23.79 μM for solasonine and from 5.01 μM to 20.68 μM for solamargine, while in healthy cell lines IC_{50} values ranged from 11.18 μM to 16.65 μM for solasonine and from 5.01 μM to 53.94 μM for solamargine. Current data on this topic suggests that both SS and SM are promising drug candidates for many diseases including melanoma.

Despite their promise, the physicochemical properties of eggplant SGAs limit their use in the treatment of skin-related diseases. Drugs for transdermal delivery should have low molecular weight (MW < 500 Da) and balanced lipophilicity (log P = 1–3) [21]. In contrast, eggplant SGAs have high molecular weight (828 Da for solamargine, and 884 Da for solasonine) [22] and unbalanced lipophilicity (log P = 5.31 for solamargine, and 4.03 for solasonine) (http://www.swissadme.ch/). Although their physicochemical properties hinder the use of these potentially highly therapeutic SGAs, there are very few studies focused on how these limitations can be overcome. Curaderm a topical cream developed for skin-related diseases, contains SS and SM in a 1:1 ratio and showed promising results in Phase I and Phase II studies. In Curaderm, transdermal delivery was increased by including urea in the formulation. In another work, Miranda et al. [23] developed a formulation via encapsulating SGA extract of *Solanum lycocarpum* into a polymeric nanoparticle. In a different study, Fe_3SO_4-solamargine magnetic liposomes were synthesized to deliver the drug for pancreatic cancer [24]. In a patent, a liposomal formulation of solamargine was developed with natural, semi-synthetic, or synthetic phospholipids, cholesterol, and an emulsifier [25].

Thus, the problem of transdermal delivery of nanocarriers containing SGA remains to be solved. In our study, liposomal formulations of SGAs were developed and characterized. In addition, liposomal formulations were tested on HaCaT and SCC-25 cell lines to assess their biological activity against skin cancer.

2. Materials and Methods

2.1. Chemicals and instrumentation

Egg yolk lecithin, cholesterol, and α-tocopherol were purchased from Applichem GmbH (Darmstadt, Germany). Natipide II, Phospholipon 80H, and Lipoid E were supplied by Lipoid AG (Steinhausen, Switzerland). Glucose, sucrose, maltose, dextrose, Ham's F12 medium, hydrocortisone, and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM)-high glucose was purchased from Gibco (Carlsbad, CA, USA). HPLC grade chloroform and methanol were purchased from VWR Chemicals (Fontenay-Sous-Bois, France). Ultrafiltration tubes were purchased from Sartorious (Goettingen, Germany). The experiments were conducted with a vacuum evaporator (Laborota 4000, Heildolph, Schwabach, Germany), an ultrasonic bath (Elmasonic S 10 H, Elma, Singen, Germany), a probe sonicator (Sonopuls, Bandelin, Germany), a NanoPlus zeta/nano particle analyser (Particulate Systems, Micromeritics, Norcross, GA, USA), a centrifuge (Allegra X-15 R, Beckman Coulter, Miami, FL, USA), a spectrophotometer (PharmaSpec UV 1700, Shimadzu, Tokyo, Japan), an HPLC (LC-20A, Shimadzu, Tokyo, Japan), a DSC (Q10, TA Instruments, New Castle, DE, USA), and an automated microplate reader (Varioskan Flash, Thermo Scientific, Waltham, MA, USA). The human keratinocyte cell line HaCaT, and the human squamous cell carcinoma cell line SCC-25 were kindly provided by Gülistan Meşe Özçivici (Department of Molecular Biology and Genetics, IZTECH, Izmir, Turkey).

2.2. Preparation of liposomes

The thin-film hydration method reported by Mengoni et al. [26] was used with some adjustments. Liposome stability is affected by the type of lecithin. Therefore, different types of lecithin were tested. These lecithins were: egg yolk lecithin which contains min. 60% phosphatidylcholine, Natipide II which is an empty-pre liposome mixture made with soybean lecithin, Phospholipon 80H which is a hydrogenated soybean lecithin with 70% phosphatidylcholine content, and Lipoid E which is an egg yolk lecithin with 70% phosphatidylcholine content. Lecithin (Applichem, Natipide II, Phospholipon 80H, or Lipoid E), cholesterol, and α-tocopherol were combined at a weight ratio of 40:6.6:1 and then dissolved in 6 mL chloroform/methanol mixture (v/v = 1:1) (Table S1). The mixture was transferred to a round bottom flask and evaporated in a rotary evaporator (150 mbar vacuum) until a thin lipid film formed (>1 h). For the hydration of the thin lipid layer, 10 mL of the appropriate hydration solvent (either 10 mM NaCl pH 4.5, 10 mM NaCl pH 5.5, PBS pH 7.4, or 10% sucrose) was added onto the thin-lipid layer in the round button flask. The resulting suspension was stirred in a shaking incubator at 150 rpm until all of the lipid film was removed from the surface of the round bottom flask. The evaporation and hydration steps were done at the same temperatures (either 40 °C, 45 °C, or 50 °C). Finally, the suspension in the round bottom flask was sonicated for 30 min at room temperature in an ultrasonic bath and then the suspension was transferred to a 15 ml falcon tube and sonicated again via a probe sonicator at 50% energy output with 5 s pulses for 2 min. Before characterization, the liposomes were allocated into three falcon tubes each of which was kept at different storage temperatures (4 °C, 25 °C, 37 °C) to evaluate their chemical and physical stability and to find the best liposomal formulation. The optimal liposomal formulation was loaded with solasonine (SS) and solamargine (SM) via passive loading. The passive loading was done with the same liposome preparation method mentioned above except 300 μM of SM and SS were added to the initial lipid-solvent mixture before evaporation. The concentration of SGAs used in the liposome formulations was decided based on a literature search of the biological activity of SGAs on different cancer cell lines.

2.3. Characterization of liposomes

Liposomes were characterized for their physicochemical properties, encapsulation efficiencies, and drug leakage, and release profiles.

2.3.1. Phase transition temperature

Phase transition temperatures of the lipids were determined via Differential Scanning Colorimetry (DSC) [27]. At least 3 mg of sample was put into an Aluminium Hermetic Pan and heated under 50 ml min^{-1} nitrogen gas flow. Heating was done with a rate of 5 °C/min from 0 °C to 100 °C (−20 °C–300 °C for Natipide II and cholesterol).

2.3.2. Physical stability

Parameters for physical stability were evaluated according to Perez

et al. [28] at different temperatures and time periods. The liposomes were aliquoted into three falcon tubes kept at different temperatures (4 °C, 25 °C, 37 °C) until visual aggregation was observed which indicates physical instability. Every seven days, samples were evaluated for size, size distribution (polydispersity index, PDI), and zeta potential properties by a zeta/nano particle analyser with a semiconductor laser ($\lambda = 660$ nm) with a scattering angle of 15° at room temperature. The diluent properties were set for a refractive index of 1.3328, for a viscosity of 0.8878 cP, and a dielectric constant of 78.3. Size measurements were done with three repetitions and 70 accumulation times. For zeta potential measurements, the same diluent properties were used. Cell constant was set to 71.310 cm^{-1} while the average voltage and the average current were set to −15.98 V/cm and −0.84 mA, respectively. Zeta potential measurements were done with three repetitions and 10 accumulation times.

2.3.3. Chemical stability

Chemical stability tests were performed each week or every two weeks from the samples used in physical stability measurements. The Stewart assay was used to determine the chemical stability of the samples [29]. Briefly, ferrothiocyanate reagent was prepared by dissolving 27.03 g ferric chloride hexahydrate and 30.4 g ammonium thiocyanate in 1 L ultra-pure water. Lipid standards were prepared in chloroform at 0.0025, 0.005, 0.01, 0.015, 0.02, and 0.025 mg/ml phospholipid concentrations. Then, 2 ml of standard or sample solution and 2 ml ferrothiocyanate reagent were mixed and vortexed for 20 s. The solutions were centrifuged at 1000 rpm for 10 min. The absorbance of the lower layer of the sample/standard solution was measured spectrophotometrically at 488 nm. Phospholipid concentrations present in the sample were calculated from the standard curve. The data were calculated as percentage stability and normalized so that day 0 measurements were equivalent to 100%.

2.3.4. Encapsulation efficiency

Encapsulation efficiency was calculated according to Amjadi et al. [30]. Specifically, 1 ml of drug-loaded liposome solution was placed into an ultrafiltration tube (MWCO = 30 KDa) and centrifuged for 40 min at 4000×*g* to separate the free drug. After centrifugation, the amount of non-encapsulated SS and SM that passed to the bottom of the tube was quantified by HPLC according to the optimized method of Eanes et al. [31], allowing indirect calculation of the encapsulated amount (Formula 1). The mobile phase for HPLC was 0.01 M Na_2HPO_4 pH 7.2 and acetonitrile (60%–40% v/v). A C18 column was used and the column temperature was set to 30 °C. The flow rate was set at 1 mL min^{-1}. The injection volume of the sample was 20 μL and UV detection was done at 200 nm. Encapsulation efficiency (EE) was calculated using Formula 2.

$$SGA_E = SGA_i - SGA_F \quad (1)$$

Where SGA_E is the encapsulated amount of SGA, SGA_i is the initial amount of SGA incorporated into liposomes, SGA_F is free SGA.

$$EE\% = \frac{\text{Amount of SGA encapsulated in liposomes}}{\text{Amount of initial SGA}} \times 100 \quad (2)$$

2.3.5. Drug leakage from liposomes

SGA leakage from liposomes was monitored over 90 d by weekly sampling according to Jiao et al. [32]. One ml of drug-loaded liposome solution was placed into an ultrafiltration tube (MWCO = 30 KDa) and centrifuged for 40 min at 4000×*g*. SGAs were quantified from the bottom of the ultrafiltration tube using HPLC as described in the previous section.

2.3.6. Drug release from liposomes

A sample and separate method was used to determine the drug release profiles of SM and SS loaded liposomes [33]. Separate falcon tubes were prepared for each time point (4, 8, 12, 16, 24, 36, 48, 60, 72 h). Release medium (acetate, pH 5.5 or PBS, pH 7.4) was added to each 15 ml falcon tube. SS and SM loaded liposomes were added to the release medium at a 1:4 ratio. The mixture was incubated in a shaking incubator at 37 °C, 100 rpm. At the end of each designated release period, a 2 ml sample was placed into an ultrafiltration tube and centrifuged for 40 min at 4000×*g*. The amount of released drug that passed to the bottom of the tube was measured using HPLC with the method described in the Encapsulation Efficiency section. Drug release kinetics of SS and SM were determined by fitting the HPLC data to the cumulative drug release model.

2.4. Anticancer activity of SGAs

2.4.1. Cell culture experiments

The anticancer activity of SS and SM were evaluated on the SCC-25 cell line with the HaCaT cell line used as healthy control. The HaCaT cells were cultured in DMEM-high glucose supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin (10,000 units of each antibiotic) in 60 mm cell culture dishes [34]. The SCC-25 cells were cultured in DMEM:F12 supplemented with 400 ng/ml hydrocortisone, 10% fetal bovine serum, and 1% penicillin-streptomycin (10,000 units of each antibiotic) in 60 mm cell culture dishes [35]. Cultures were kept in an incubator with 5% CO_2 at 37 °C. During treatments and assays, all relevant drugs and solutions were dissolved or diluted in the appropriate medium for the cells.

2.4.2. Cell viability assay

The MTT assay protocol that was described by Mossman was used with slight modifications to evaluate the cytotoxic activities of solasonine and solamargine [36]. Cells were seeded in 48-well plates (2×10^4 cells per well) or 96-well plates (1×10^4 cells per well) with the appropriate medium. After 24 h incubation, the medium was removed and replaced with medium supplemented with free SS or/and SM or liposome-encapsulated SS and SM at predetermined concentrations. Medium only and MeOH (for free SS and SM treatment) or empty liposomes (for liposome encapsulated SS and SM treatment) were used as negative control groups. After 24 h of incubation with treatment materials, the medium was removed and replaced with MTT solution (5 mg/ml) diluted in the medium at a ratio of 1:10. Cells were incubated with MTT solution for 4 h at 37 °C. After incubation, plates were centrifuged at 1800 rpm for 10 min. Finally, the culture medium and MTT solution were removed and replaced with 150 μL (for 96-well plate) or 300 μL (for 48-well plate) dimethyl sulfoxide (DMSO). Plates were wrapped with aluminium foil and put on an orbital shaker at 300 rpm for 5–10 min until formazan crystals were completely dissolved. The absorbance of samples was measured at 570 nm with an automated microplate reader. The results were calculated as cell viability relative to the appropriate negative control.

2.4.3. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, San Diego, CA, USA). Unless stated otherwise, standard deviations were calculated with three different measurements of the same sample. All cell culture experiments were conducted at least twice and with at least three technical replicates. All the measurements were compared to the control samples and were statistically analysed with the two-way ANOVA test with Dunnett's test for multiple comparisons. Differences were considered statistically significant when $p < 0.05$ (*, #).

3. Results

3.1. Preparation of liposomes

3.1.1. Hydration temperature

Phase transition temperatures (Tm) of the phospholipids were

measured via DSC. Applichem and Lipoid E lecithin had similar transition temperatures: 10.83 °C and 10.13 °C, while Natipide II and Phospholipon 80H lecithin had much higher transition temperatures of 91.09 °C and 71.34 °C respectively (Table 1). We also measured the Tm of cholesterol which was 148 °C (Table 1).

3.1.2. Hydration solution

Three different hydration solutions that were most frequently encountered in the literature were tested: NaCl, PBS, and sucrose. Average sizes of the liposomes created with NaCl pH 4.5, NaCl pH 5.5, PBS pH 7.4, and 10% sucrose were 114.6 nm, 110.4 nm, 348.2 nm, and 148.4 nm, respectively (Fig. 1). Polydispersity Index (PDI) values of these liposomes ranged from 0.25 to 0.28.

3.1.3. Physiochemical properties of candidate formulations

Each lecithin was used in three different liposomal batches at varying hydration temperatures (40 °C, 45 °C, and 50 °C). The resultant liposomes were allocated to three different storage temperatures (4 °C, 25 °C, and 37 °C) to determine optimal conditions. Physical and chemical stabilities were monitored until visible aggregation was detected in the liposomal solution. Physical and chemical instability were observed relatively quickly in liposomal solutions prepared with Lipoid E at hydration temperatures of 40 °C and 50 °C and with Phospholipon 80 H at hydration temperatures of 45 and 50 °C. Thus, the stability experiments were conducted with Lipoid E and Phospholipon 80H at hydration temperatures of 45 °C and 40 °C, respectively.

Initial average sizes of Applichem liposomes prepared at 40 °C, 45 °C, and 50 °C were 109.1 nm, 110.2 nm, and 99.7 nm (Fig. 2A, D, G) while initial average sizes of Natipide II liposomes were 89.8 nm, 87.6 nm, and 87.2 nm for the same hydration temperatures (Fig. 2A, D, G). PDI values of Applichem liposomes varied only slightly (from 0.25 to 0.26) at these temperatures; while the PDI values of Natipide II liposomes were 0.27, regardless of storage temperature. The physical stabilities of both groups of liposomes were protected throughout the study at 4 °C storage (Fig. 2A, D, G). At 25 °C storage, however, the average size of the liposomes started to increase on day 35. At the end of the study, Applichem liposomes were at least 2060 nm in diameter while Natipide II liposomes were at least 212.7 nm (Fig. 2B, E, H). At 37 °C storage, Applichem liposomes shrank throughout the study and dropped to an average diameter of 96.5 nm, regardless of hydration temperature (Fig. 2C, F, I). Throughout incubation, PDI values of all samples remained less than 0.5 regardless of lecithin type, hydration, and storage temperatures.

Initial average sizes of Lipoid E and Phospholipon 80H liposomes were 118.27 nm and 116 nm with PDI values of 0.28 and 0.22, respectively (Fig. 2A, D). Physical stabilities of these liposomes were protected at 4 °C storage with average liposome sizes of 124.7 nm and 118.6 nm and PDI values that were unchanged (Fig. 2A, D). At 25 °C storage, the physical stability of Lipoid E liposomes was preserved with an average size of 123.7 nm on day 105 (Fig. 2E). However, at this temperature, Phospholipon 80H liposomes gradually lost their physical stability. Loss of physical stability started on day 21 and reached the maximum average size of 985.06 nm on day 56 (Fig. 2B). At 37 °C storage, both Lipoid E and Phospholipon 80H slightly lost their physical stabilities with average sizes of 103.7 nm and 148.4 nm, respectively, on day 56 (Fig. 2C, F). PDI values of these liposomes were below 0.5

Table 1
Phase transition temperatures of lipids.

Lipid	Phase Transition Temperature T(m) °C
Applichem	10.83
Natipide II	91.09
Phospholipon 80H	71.34
Lipoid E	10.13
Cholesterol	148

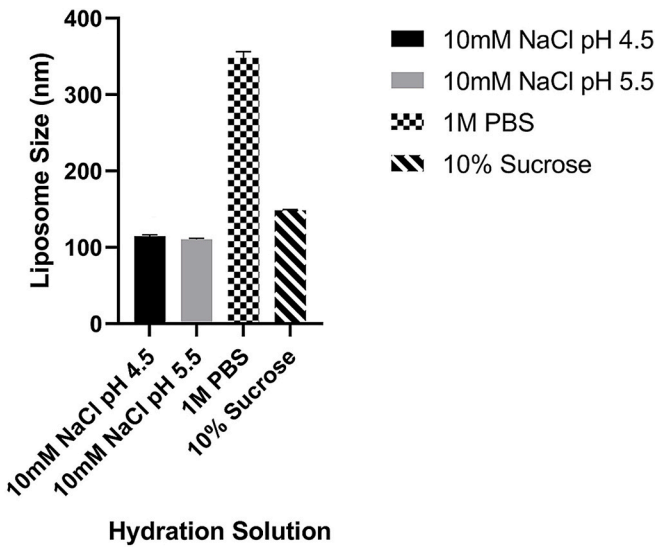

Fig. 1. Size comparison of liposomes prepared with different hydration solutions.

regardless of lecithin type and storage temperature.

The chemical stability of the samples was determined via the Stewart Assay. Chemical stability was protected in all Applichem liposomes stored at 4 °C regardless of their hydration temperature (Fig. 3A, D, G). However, when stored at 25 °C, the chemical stability of Applichem liposomes dropped to a minimum of 12% by day 35 (Fig. 3E). When stored at 37 °C, the chemical stability of the liposomes was slightly better preserved with a minimum stability of 40% on day 49 (Fig. 3C, F, I). The chemical stability of Natipide II liposomes varied considerably depending on hydration and storage temperatures. Natipide liposomes stored at 4 °C had stabilities that dropped to 75–85% and remained at those levels for the rest of the study (Fig. 3A, D, G). When stored at 25 °C, the chemical stability of the Natipide liposomes dropped to a minimum of 47% on day 28 (Fig. 3E). At 37 °C storage, the Natipide liposomes' chemical stability dropped to a minimum of 39% on day 49 (Fig. 3C, F, I). The chemical stabilities of the Lipoid E and Phopholipon 80H liposomes were protected throughout 112 days when stored at 4 °C (Fig. 3A, C). At 25 °C however, Phospholipon 80H liposomes gradually lost their chemical stability with a minimum of 66% stability on day 66 (Fig. 3B) while Lipoid E liposomes protected their chemical integrity almost completely, with a minimum of 92% stability on day 66 (Fig. 3E). At 37 °C storage, both Lipoid E and Phospholipon 80H liposomes gradually lost their chemical stabilities and ended with 74% and 72% stabilities, respectively (Fig. 3C, F).

3.2. Characterization of drug loaded liposomes

3.2.1. Physiochemical properties

Physical stabilities of the loaded liposomes were monitored by their size and zeta potential and were mostly preserved. Loaded liposomes averaged 146.5 nm in diameter at the beginning of the experiment and increased to 211.83 nm at the end of the 3-month monitoring period (Fig. 4A). The PDI value of the liposomes started at 0.24 and constantly decreased to 0.16 by day 90. Throughout the monitoring period, the zeta potential of loaded liposomes fluctuated around −20 mV (Fig. 4B).

The chemical stabilities of the loaded liposomes were determined as percent stability as described earlier. As with the empty liposomes, the chemical stability of loaded liposomes was preserved throughout monitoring (Fig. 4C).

3.2.2. Encapsulation efficiency

Following the production of loaded liposomes, ultrafiltration tubes

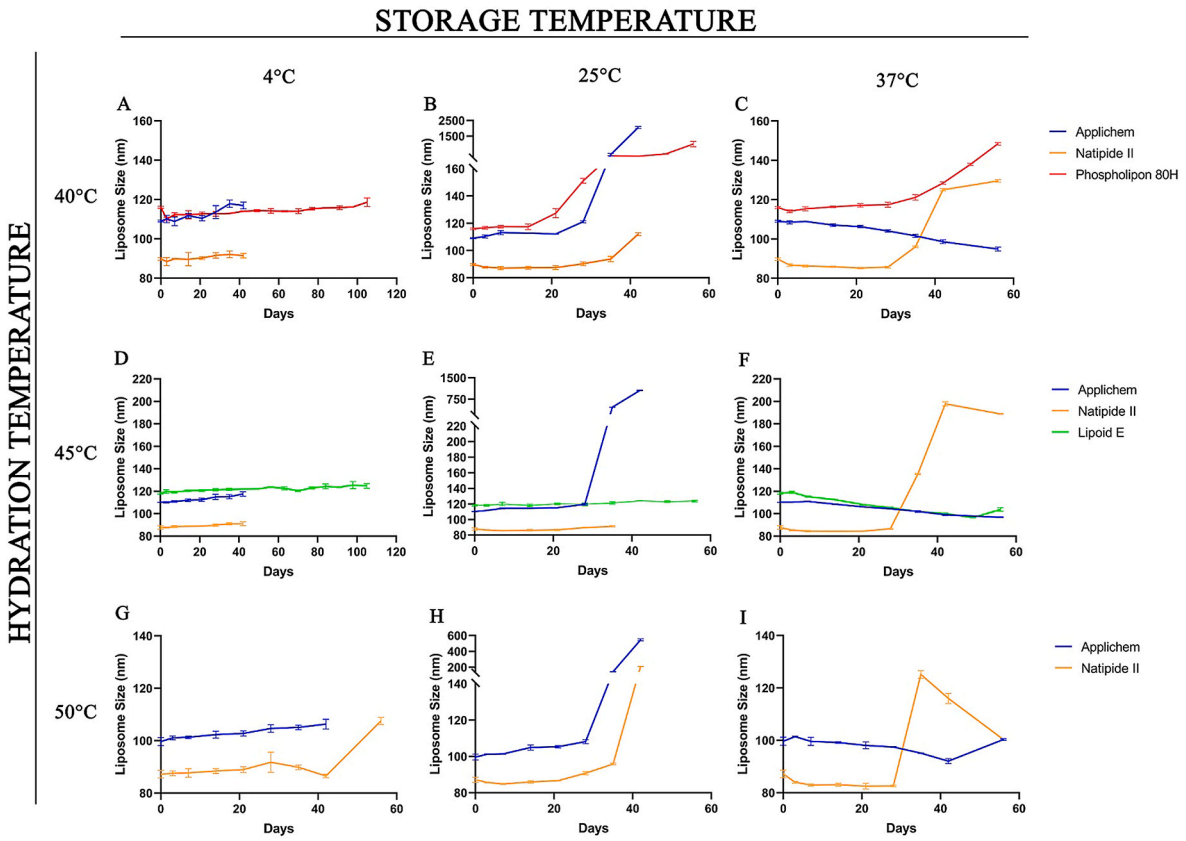

Fig. 2. Physical stability monitoring of Applichem, Natipide II, Phospholipon 80H, and Lipoid E liposomes via DLS. Liposomes that were prepared at 40 °C (A–C), 45 °C (D–F) and 50 °C (G–I) hydration temperatures were allocated for storage at 4 °C, 25 °C, and 37 °C. Physical stability monitoring was done for 2 or 4 months, however, in some samples, monitoring was halted as soon as precipitation in the sample was observed.

were used to separate unencapsulated SS and SM from loaded liposomes. Unencapsulated SS and SM were quantified via HPLC. Encapsulation efficiencies for loaded liposomes were 84.5% for SS and 89.7% for SM (Fig. 5).

3.2.3. Drug leakage from liposomes

Drug leakage from loaded liposomes was monitored for three months to assess the stability of the loaded liposomes. Leaked SS and SM were again separated with ultrafiltration tubes and measured with HPLC. A constant, small amount of leakage was detected throughout the monitoring. At the end of three months, only 10% of SS and 8% of SM had leaked from the liposomes (Fig. 5).

3.2.4. Drug release from liposomes

To determine the release profiles of the loaded liposomes, two different release media were used: acetate buffer pH 5.5 and PBS pH 7.4. In acetate buffer, the release of SS and SM started with an initial outburst at hours 10 to 20 during which 20% of SM and 15% of SS were released. After 20 h, this release stopped. (Fig. 6A). During the same time period, SS and SM release was only 5% in PBS pH 7.4. After the initial burst, drug release also ceased in this buffer (Fig. 6B).

In an additional trial, 10% methanol was used as a destabilizing agent in the PBS release medium. The release was monitored for 24 h. A total of 50% of the drug load was released within the first 4 h as an initial burst then release was suspended (Fig. 6C).

Individual stability of SS and SM at 37 °C must also be determined to evaluate our data. The stability of SS and SM did not change during a week of incubation at 37 °C (Fig. 6D).

3.3. Anticancer activity of SGAs

In order to test the biological effects of SS and SM, skin-related squamous cell lines HaCaT and SCC-25 were used. HaCaT is a healthy immortalized human keratinocyte cell line and SCC-25 is a human tongue squamous cell carcinoma line. According to the results, SCC-25 is less susceptible to SS and SM than HaCaT (Fig. 7). The IC_{50} values for the HaCaT cell line were 10.17 μM for free SS, 1.975 μM for free SM (Figs. 7A), and 1.52 μM when both SGAs were combined (Fig. 7C). IC_{50} values for the SCC-25 cell line, however, were a bit higher: 25.83 μM for SS, 9.676 μM for SM (Figs. 7B), and 3.528 μM for SS/SM combined (Fig. 7C). When SS and SM were encapsulated in a liposome (300 μM SS + 300 μM SM) the IC_{50} values jumped to 182.9 μM for SCC-25 and to 101.1 μM for HaCaT (Fig. 7D).

4. Discussion

Eggplant SGAs are important phytochemicals with diverse bioactivities. The factors limiting the use of alkaloids as drugs are their high molecular weight, amphiphilic nature, low bioavailability, and poor water solubility [37]. Thus, it is important to develop a formulation that overcomes these limitations. Liposomal formulations can be a good choice to increase the transdermal delivery of SGAs. An optimal liposomal formulation should protect physical and chemical stability for a long time, provide a high degree of encapsulation, and release the drug in a slow and sustained manner at the target site. Given these advantages, liposomal formulations of SGAs deserve further study. Therefore, we developed stable liposomal formulations of SM, SS, and both and optimized our formulation according to parameters that affect the stability, encapsulation, and release properties of the liposomes.

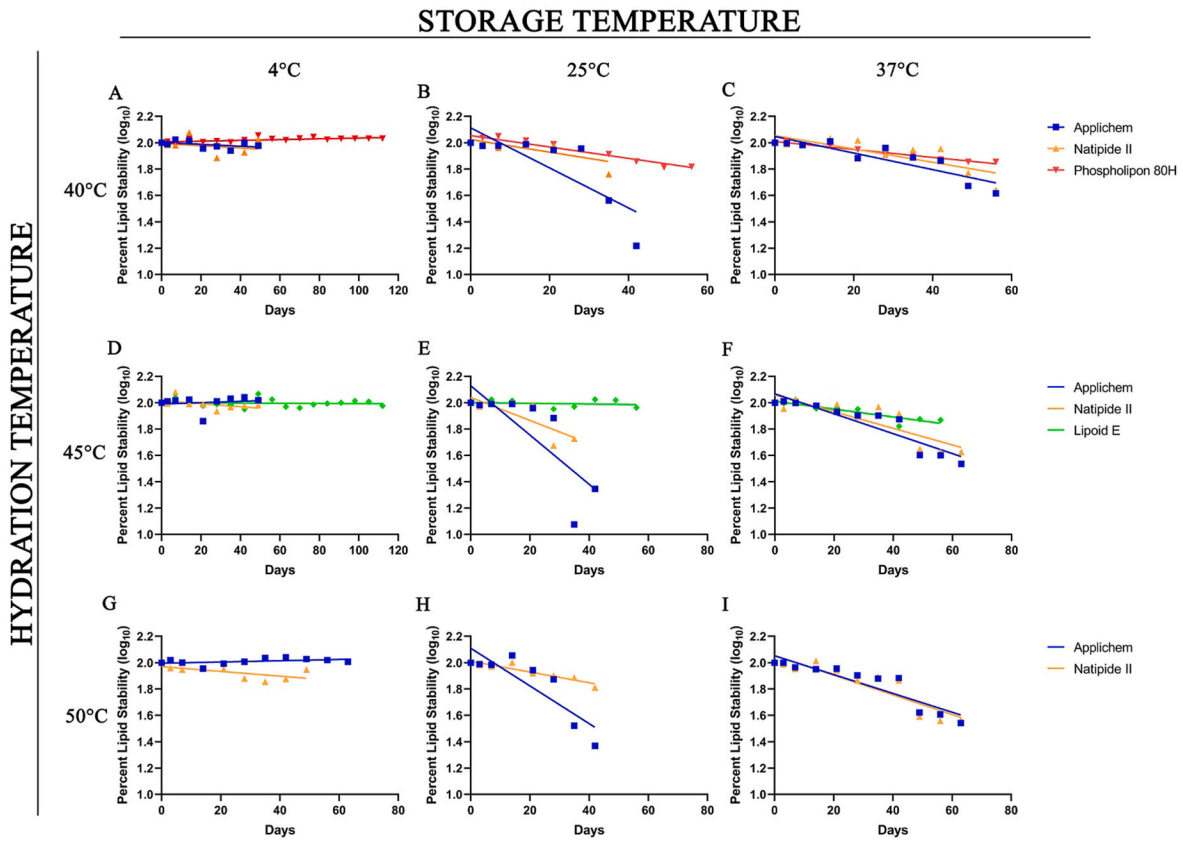

Fig. 3. Chemical stability monitoring of Applichem, Natipide II, Phospholipon 80H, and Lipoid E liposomes via Stewart Assay. Liposomes that were prepared at 40 °C (A–C), 45 °C (D–F) and 50 °C (G–I) hydration temperatures were allocated for storage at 4 °C, 25 °C, 37 °C. Chemical stability monitoring was done for 2 or 4 months however, in some samples, monitoring was halted as soon as precipitation in the sample was observed.

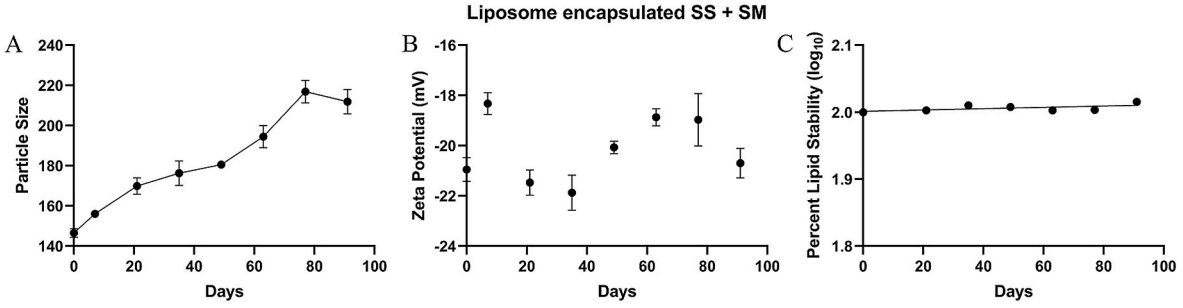

Fig. 4. (A) Change in size of SS + SM loaded liposomes over three months (B) Change in the zeta potential of SS + SM loaded liposomes over three months (C) Chemical stability of SS + SM loaded liposomes over three months.

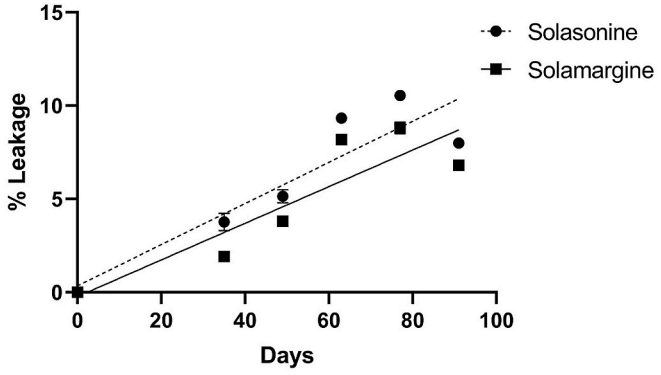

Fig. 5. Percent leakage of SS and SM from loaded liposomes.

4.1. Liposome preparation

4.1.1. Hydration temperature

Hydration temperature is one of several factors that affect liposome encapsulation efficiency. As the temperature approaches the phase transition temperature of the phospholipid, the phospholipid bilayer becomes less rigid, increasing its permeability and, thus, affecting drug encapsulation [38,39]. For this reason, it is recommended that the hydration temperature used to prepare liposomes should be higher than the phospholipid's phase transition temperature (Tm) [40]. In our measurements both Applichem and Lipoid E showed similar transition temperatures at around 10 °C as we expected due to their similar chemical content while Natipide II and Phospholipon 80H lecithin had transition temperatures higher than 70 °C (Table 1). Knowledge of the phospholipid's T_m is not sufficient to determine the liposome's

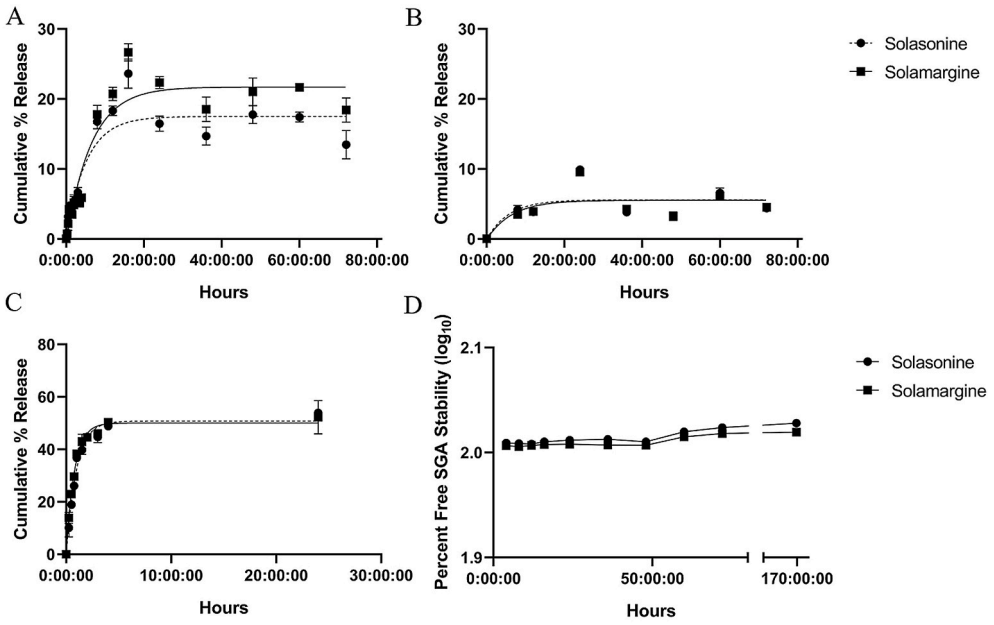

Fig. 6. The release profile of SS + SM from liposomes (A) in acetate buffer, pH 5.5; (B) in PBS, pH 7.4; (C) in acetate buffer, pH 5.5; supplemented with 10% methanol (D) percent stability of SS and SM in their free forms.

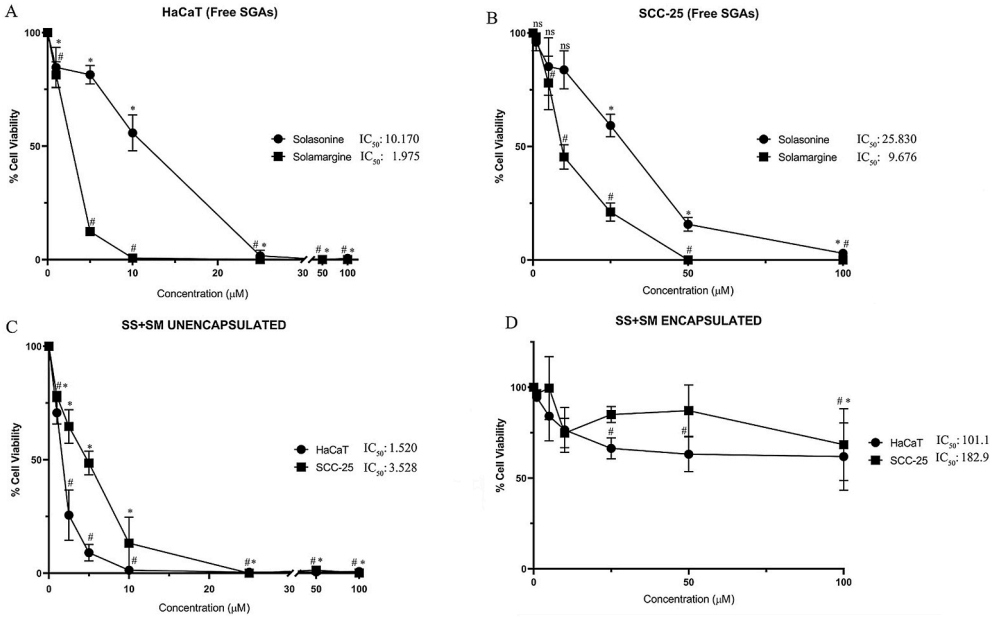

Fig. 7. Dose-dependent changes in cell viability over 24 h for free SS or free SM in (A) HaCaT cell line, (B) SCC-25 cell line. Dose-dependent changes in cell viability over 24 h for (C) unencapsulated SS and SM and (D) encapsulated SS and SM for 24 h in HaCaT and SCC-25 cell lines. Statistical differences are indicated with * (solamargine treated vs control) and # (solasonine treated vs control), $p \leq 0.05$.

hydration temperature because the T_m does not represent the temperature at which the liposomal structure becomes less rigid. Indeed, there are reports that liposomes reach phase transition at temperatures lower than the phospholipid T_m [41,42]. This situation might be explained by the presence of cholesterol in the formulation which lowers the phase transition temperature of the liposomes [43]. Since our formulation contains cholesterol and many previous studies used hydration temperatures between 40 °C and 60 °C [44–47], three different hydration temperatures (40 °C, 45 °C, 50 °C) were tested in liposome formulations. By assessing the liposomes' physiochemical properties, the most suitable hydration temperature for each lecithin type was selected for final formulations as described in the Physical Stability section.

4.1.2. *Hydration solution*

Hydration solution is another key factor that has a significant effect on the physiochemical properties of liposomes. Hydration solution affects many features of liposomes including their stability [48], size, and encapsulation efficiency [49–51]. We tested NaCl at pH 4.5 and 5.5, PBS at pH 7.4, and 10% sucrose as hydration solutions. For all but PBS (348.2 nm), the liposomes were smaller than 200 nm (Fig. 1) with similar PDI values (0.26, on average) and no statistically significant differences between the samples for both size and PDI value. Because the PDI values of all samples were lower than 0.5 indicating that all liposomes were uniform, we focused on liposome size to choose the best possible hydration solution. Similar to our findings in size

measurements, comparative studies in the literature indicated that the use of PBS as a hydration buffer resulted in larger liposomes compared to 10% sucrose and 0.9% saline buffers [52]. Also when compared with other hydration solutions, the saline buffer showed better encapsulation efficiency with lower concentrations of saline providing greater efficiency [53]. On the other hand, smaller carriers are better vehicles for drug delivery across skin layers [54]. Thus, based on our results and previous studies, NaCl was preferred to sucrose and PBS. Moreover, because the skin surface pH is generally estimated as 5.4–5.9 with pH 5.5 recommended to achieve high yields from skin products [55], we chose the pH 5.5 NaCl solution for hydration. This solution is not expected to create any immunogenic reactions as its pH is optimal and NaCl concentration is low (10 mM).

4.1.3. Physiochemical properties of candidate formulations

Phospholipids are the main component of liposomal structures, therefore the type of lecithin used in liposome production has a direct effect on their stability in terms of both physical and chemical properties [56,57]. We tested four lecithins of different origins as described in detail in the Materials and Method section. As mentioned earlier, three different hydration temperatures (40 °C, 45 °C, and 50 °C) were tested for detecting the optimal one for each lecithin.

Regardless of lecithin type or hydration temperature, the liposomes were smaller than 150 nm (between 87.2 and 118.27 nm) which is desirable for skin applications [54] (Figs. 2–4). All PDI values were around 0.25, confirming uniformity. The physical stability of the liposomes was protected for at least 8 weeks for Applichem and Natipide II liposomes and at least 16 weeks for Phospholipon 80H and Lipoid E liposomes at 4 °C storage regardless of hydration temperature and lecithin type (Fig. 2A, D, G). This result was expected as similar liposomal formulations did not show a change in size at storage temperatures lower than 8 °C [58–62]. However, stability in liposome size which is an indication of physical stability, lasted for a maximum of one month in most of these formulations [59–61].

On the other hand, at 25 °C storage, Applichem and Phospholipon 80H liposomes gradually lost their physical stability with the change starting on day 21 (Fig. 2B, E, H). The phase transition temperatures of these two liposomes might explain their instability at 25 °C. It is recommended that liposomes be stored at much higher or lower temperatures than their phase transition temperatures because, at those temperatures, they are more prone to bilayer defects that lead to physical instabilities [63]. Until the 5th week of incubation at 37 °C, the sizes of the liposomes did not change similar to the findings of Elsana et al. in which an empty liposome formulation stored at 37 °C did not show a significant size change until the 5th week [58]. (Fig. 2C, F, I). The rate of stability loss in that study, however, was much faster than observed in our formulations. Throughout the stability tests, all liposomes protected their uniformity regardless of lecithin type, hydration, or storage temperature as PDI values of all samples remained less than 0.5.

To the best of our knowledge, this is the first study to use the Stewart Assay to determine the long-term chemical stability of liposomes. Similar to physical stability, the chemical stability of all liposomes was protected at 4 °C regardless of their hydration temperature and lecithin type (Fig. 3A, D, G). At 25 °C however, Phospholipon 80H liposomes protected their chemical stability despite having lost their physical stabilities. This inconsistency between physical and chemical stabilities can be explained by factors that can trigger aggregation like pH and ionic strength of the solution. Similar to the physical stability results, Applichem liposomes lost their chemical stability while Natipide II liposomes were not considerably altered (Fig. 3). At 37 °C, all liposomes regardless of hydration temperature and lecithin type lost their chemical stabilities while substantially protecting their physical stabilities (Fig. 3C, F, I). From these results, it can be suggested that there is no direct relationship between physical and chemical stabilities when storage temperature is considered.

4.2. Characterization of drug-loaded liposomes

After evaluating the stability data, Phospholipon 80H was selected for the loaded liposomal formulation. New batches of SS and SM loaded Phospholipon 80H liposomes were prepared and monitored for 90 days. These liposomes were stored at 4 °C as it was the best storage temperature in terms of protecting the physical and chemical stabilities of the liposomes.

4.2.1. Physiochemical properties

As mentioned earlier, SS and SM are amphiphilic molecules and are expected to be located in the lipid bilayer. This might change some characteristics of the liposomes. Taking this into consideration, the physical and chemical stabilities of drug-loaded liposomes were monitored for three months. For these comparisons, liposomes made of Phospholipon 80H were loaded with 300 μM SS and 300 μM SM and stored at 4 °C. Initial average sizes of the drug-loaded liposomes were less than 150 nm which is desirable as smaller carrier complexes have a better chance to pass through the skin barrier. This diameter also falls within the range seen in many commercial antitumor drug liposomal formulations (Doxil©, Myocet©, Marqibo©, Onivyde©) which vary from 80 nm to 250 nm [64]. During three months of storage, the liposomes enlarged by only 65 nm indicating that physical stability was well preserved (Fig. 4A, Table S1). Thus, the liposome size remained within the range acceptable for skin penetration. The PDI value also stayed in an acceptable range (max. 0.24) (Table S1). The chemical stability of the loaded liposomes was also protected completely during the three months (Fig. 4C). Another parameter that affects liposome stability is zeta potential. It is reported that the electrostatic repulsion created by ± 20 mV potential prevents the aggregation of liposomes to some degree [65]. Throughout the monitoring period, the zeta potential of the loaded liposomes fluctuated around −20 mV (Fig. 4B, Table S1), which might help explain the high physical stability of our liposomal formulation. Due to time constraints, stability monitoring was limited to three months demonstrating that our formulation is stable for *at least* this time period. There is much variation in liposomal formulation stability reported in the literature. While some formulations are stable for less than a month [59,60], commercially available Doxil© has stability for at least 6 months [66]. Thus, we conclude that our formulation has good physiochemical stability, however, this stability must be monitored over a longer storage period. In addition, methods like lyophilization can be tested to extend storage stability.

4.2.2. Encapsulation efficiency

Encapsulation efficiency is a major determinant of a successful liposomal formulation. Encapsulation efficiencies for the loaded liposomes were 84.5% for SS and 89.7% for SM. Although there are no previous data in the literature about the efficiency of SGA encapsulation by liposomes, comparison with other natural, amphiphilic compounds can give an idea of the encapsulation efficiency of SGAs. For example, lutein [67] and eugenol [68] were encapsulated in supercritical CO_2-assisted liposome preparations with an encapsulation efficiency ranging from 56.7 to 97.0% and 80.4–94.2%, respectively, depending on the preparation parameters. In another study, the encapsulation efficiency of eugenol varied between 77.0 and 94.4% depending on the phospholipid type [69]. The commercial liposomes Doxil©, and Onivyde© have encapsulation efficiencies of 90% and higher [64,66]. Thus, encapsulation of more than 85% of the drugs into liposomes was an indicator of the high efficiency achieved in our study.

4.2.3. Drug leakage

Leakage of the liposome's contents is an important parameter affecting liposome stability. The encapsulated drug should be preserved in its encapsulated form until it is taken up by the target site [70]. In this study, drug leakage from loaded liposomes was monitored for three months. A constant, low level of leakage was detected throughout

monitoring, however, at the end of three months, only 10% of SS and 8% of SM were lost by leakage (Fig. 5). Again, these data indicate the high stability of our liposomal formulation. In a study with Pirfenidone, 18% of the drug was leaked in just one week [59]. In another study, 30% of the drug was leaked in the first seven days, and drug leakage rose to 70% at day 40 [71]. In our formulation, SS and SM leaked at similar rates meaning that neither drug was chemically favoured by the liposomes. This is probably due to the similar chemical structures of SS and SM.

4.2.4. Drug release

The release profile of a carrier system is crucial to avoid the 'peaks and valleys' that are seen in the release profile of conventional drugs [72]. To avoid such peaks and valleys, a carrier system should constantly release its contents. To determine the release profile of the loaded liposomes, we used two different release media: acetate buffer (pH 5.5) and PBS (pH 7.4). These two media were used to mimic the pH values of skin and cell culture environments, respectively. Compared to PBS, three to four times more SS and SM were released in acetate buffer meaning that our liposomal formulation released better at a pH value similar to that of skin (Fig. 6A and B). Liposome stability and the physical and chemical properties of the release environment affect the drug release rate. In our release buffers, we could only imitate the temperature and pH of the release environment. But it must be noted that liposomal drug release is also affected by lipid-lipid interactions between the liposomes and the skin SC that can cause the liposomes to lose integrity and increase drug release. Moreover, the liposomes were developed and intended for topical use. Therefore, environmental effects like sunlight and humidity must be taken into consideration while determining the release profile. Thus, 10% methanol was used as a destabilizing agent in the release medium, and then, the release was monitored for 24 h [73]. Under these conditions, almost half of the drug load was released within the first 4 h as an initial burst followed by no further release (Fig. 6C). The slow release of the liposomes might be due to chemical interactions between SGAs and sterols, especially interactions between solamargine and cholesterol [74]. This strong interaction might not only result in slow release but also provide structural support for the liposome itself. In this case, release rates can be adjusted by decreasing the amount of cholesterol, solamargine, or both.

These results indicate that, once in contact with skin, the liposomes may release much more of their drug content, however, further tests must be conducted with animals to obtain a more reliable release profile. Because release was higher in acetate buffer than PBS, our results suggest that loaded liposomes were more sensitive to an acidic environment. Similar release results are reported in the literature. In a study with Vancomycin and Rifampin, 50% and 23% of drug contents were released in the first 4 h, respectively. However, the complete release was not achieved until 13 days for Vancomycin and 7 days for Rifampin [75]. In another study, curcumin release was monitored daily. Within 24 h, 14% of the drug was released with 70% released after 48 h. The complete release occurred in 10 days [76]. In a study with Doxil©, less than 3% of the drug was released in the first 4 h. At 96 h only 38% of the drug was released [66].

Individual stability of SS and SM in the release medium (at 37 °C) was evaluated to validate the data and to assess free drug stability. The stability of SS and SM did not change during 7 days of incubation at 37 °C which indicated that SS and SM would not be degraded during the release period (Fig. 6D).

4.3. Anticancer activity of SGAs

As mentioned in the introduction although many cell lines have been treated with SS and SM, not many skin-related cells or tissues were tested with these drugs. To test the biological effects of SS and SM, healthy and non-melanoma keratinocyte cell lines HaCaT and SCC-25 were used. According to the results of our cell culture experiments, SCC-25 cells are less susceptible to SS and SM than HaCaT cells as the IC_{50} values for HaCaT cells were lower indicating greater toxicity (Fig. 7). Although both cell lines are keratinocytes in origin they are originated from different tissues. Therefore, more definitive experiments would involve HaCaT compared with human skin non-melanoma cells and melanoma cells. In the absence of such results, we can compare our IC_{50} values with those in the literature. SS and SM were tested on the mouse skin melanoma cell line B16–F10 and their IC_{50} values were 24.19 μM and 10.15 μM, respectively, which is similar to the results obtained with SCC-25 (Fig. 7). These results suggest that the free form of SM is much more toxic than that of SS. When applied together, SM and SS demonstrated a synergetic effect in SCC-25 but not in HaCaT. This suggests that SS and SM may have different mechanisms of action in SCC-25 cells.

Encapsulated SS and SM had very low toxicity in both SCC-25 and HaCaT cell lines (Fig. 7D). This reduction in efficacy can be explained by the drug release profile data. In PBS (pH 7.4), loaded liposomes were able to release only 5% of their drug content within the first 20 h. Therefore, the IC_{50} values do not represent the actual concentration of the drug in the culture. Also, it is reported that liposomal applications of MTT incorrectly show higher viability [77]. Thus, the actual IC_{50} value is most likely much lower than that observed during the experiments. Considering all this information, further experiments must be conducted with both cell lines and animals to estimate more accurately the release profiles and biological activities of SS and SM. New cell culture experiments would also help elucidate the mechanisms of action of SS and SM. The results of such studies would be beneficial to develop a new liposomal formulation with optimized amounts of SS and SM.

5. Conclusion

This is the first report describing liposomal formulations of SGAs. Liposomes prepared with Phospholipon 80H were most stable (about four months at 4 °C) compared with other liposomal formulations. The formulation developed in this study had high encapsulation efficiency, very low drug leakage, and was stable over 3 months at 4 °C. Thus, this liposomal formulation is promising not only for SS and SM but also for other amphiphilic drugs. In cell culture, the MTT data showed that SM was a more efficient anticancer agent than SS. Further experiments are needed with animals to obtain better release profile and toxicity data. Furthermore, these molecules and their liposomal formulations can be tested with other skin melanoma cell lines and against other skin-related diseases like leishmaniasis. Such studies will help reveal the mechanism of action of SS and SM so that new liposomal formulations can be developed in a disease or patient-specific manner.

Author statement

Engin Tatlidil: Investigation, Formal Analysis, Writing—Original Draft. **Nergiz Gurbuz Colak**: Conceptualization, Methodology, Writing—Review & Editing. **Sami Doganlar**: Resources, Supervision. **Anne Frary**: Supervision, Writing—Review & Editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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