Original Article

DOI: 10.5582/ddt.2024.01018

Antioxidant activity of *Sophora exigua* and liposome development of its powerful extract

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SUMMARY *Sophora exigua* (SE) was sequentially extracted using hexane, ethyl acetate, and ethanol. The obtained extracts were tested for antioxidant activity. Among them, the fractionated ethyl acetate extract (SE-EA) showed the highest potential in free radical scavenging and ferric-reducing properties. The chemical analysis identified sophoraflavanone G as one of the active ingredients in SE-EA. According to SE-EA solubility, SE-EA liposomes were developed using a sonication-assisted thin film method. Cholesterol and phospholipids were used as the main compositions of the liposomes. The obtained liposomes were spherical with different nano-size ranges, size distribution, and zeta potential depending on SE-EA and total lipid concentrations. SE-EA liposomes were slightly bigger than their empty liposomes. All liposomes exhibited a phospholipid crystalline structure. Cholesterol and SE-EA existed in the liposomes as an amorphous state. SE-EA liposomes with high total lipid content exhibited high entrapment efficiency and sustained release behavior. Whereas liposomes with low total lipid content showed low entrapment efficiency and fast-release behavior. All SE-EA liposomes showed stronger antioxidant activity than the non-entrapped SE-EA. In conclusion, SE-EA is a natural source of potent antioxidants. The developed SE-EA liposomes are a promising pharmaceutical formulation to efficiently deliver the active ingredients of SE-EA and are suitable for further study *in vivo*.

Keywords *Sophora exigua*, free radical scavenging, reducing power, film method, liposomes

1. Introduction

Medicinal plants have been historically used for treatment of several diseases in humans. *Sophora exigua* Craib, is one of the most potential medicinal plants in Asian traditional remedies. This plant belongs to the Fabaceae family. Biological activities of *Sophora* species have been of interest and studied for several years (*1*). These include antibacterial, antifungal, antiviral, and anticancer activities as well as an effect on hair growth (*2*). *S. exigua* is widely found in Asian countries especially Thailand and Cambodia. The root of *S. exigua* is an important component in a Thai traditional medicine called "Kheaw-Hom" which is used as an antipyretic and anti-inflammatory agent (*3*). The extract of *S. exigua* root have been reported to have antioxidant, and antimalarial activities (*4,5*). It has been reported that flavonoid compounds extracted from *Sophora* species exhibited antimicrobial activities against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Candida albicans* (*6*). The flavone compounds extracted from *S. exigua* have high activity on reduction of bacterial membrane fluidity and inhibition of methicillin-resistant *Staphylococcus aureus* (*7,8*). Recently, the ethyl acetate extract of *S. exigua* root have been demonstrated to have anti lung cancer through NLRP3 inflammasome pathway inhibition (*9*). Antioxidant activity of plants in the same genus such as *S. flavescens* has been reported (*10*). However, there was no report on antioxidant activity of *S. exigua*.

Using potential medicinal plant extracts for treatment of many diseases is increasing interest since they are natural and environmentally friendly. Chemical agents, *e.g.*, chemotherapeutic drugs always affect both cancer and normal cells and cause several severe

side effects including hair loss, nausea, vomiting, and diarrhea (*11*). Medicinal plants are, therefore, increasing investigated for cancer treatment and prevent toxicity to normal cells (*12*). However, clinical applications of plant extracts are limited due to their low solubility and permeability leading to low bioavailability in biological system (*13*). Suitable formulations are needed to improve their bioavailability and therapeutic impact. Several approaches, including nano delivery systems, have been applied to solve these problems (*14,15*). Among the nano delivery systems, liposomes are one of the most effective systems because they can entrap the insoluble drugs into their nano-sized structure and modify the *in vivo* behavior to reduce toxicity (*16*). In addition, liposomes are biocompatible enough to be approved for parenteral administration (*17*).

Liposomes are an excellent delivery system for enhancing drug bioavailability. The hydrophilic structure of liposomes can entrap hydrophilic drugs in the aqueous core while the water insoluble molecules can be incorporated in their lipid bilayer structures. The most suitable formula of liposomes can provide the systems with increasing bioavailability, preventing degradation, and increasing efficacy of active substances as well as reducing toxicity (*18*). This study focuses on investigating the antioxidant activity of *S. exigua* fractionated from various solvents and the development of liposomes of the most potent extract of *S. exigua*.

2. Materials and Methods

2.1. Chemicals and plant materials

Ferrous sulfate and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), potassium persulfate, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2'-azinobis-(3- ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT), ferric chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, methanol, chloroform, ethyl acetate, and n-hexane were purchased from RCI Labscan (Bangkok, Thailand). Pentylene glycol was supplied by Forecus (Bangkok, Thailand). Phosphate buffer solution was purchased from Calbiochem (San Diego, CA, USA). Triton X-100 was from Loba Chemie (Mumbai, India). Phospholipids (phosphatidylcholine) from hydrogenated lecithin and cholesterol were purchase from Chanjao Longevity (Bangkok, Thailand). Other chemicals and solvents are of the highest grade available.

S. exigua were collected from Bangkok, Thailand during April 2020. The plant material was identified and authenticated by a botanist (Dr. Angkhana Inta) of Chiang Mai University. A voucher with specimen number WP6612 has been deposited in the Herbarium, Queen Sirikit Botanical Garden.

2.2. Extract preparation and standardization

Dried root of *S. exigua* was ground into a fine powder. Then, the dried powder was fractionated extracted by macerating with n-hexane for 3 cycles at room temperature. The macerating period for each cycle was 24 h. During maceration, the mixture was stirred using a magnetic stirrer at 100 rpm. After each cycle, the mixture was filtered, and the filtrates were pooled together. The solid residue was dried under open air and macerated with ethyl acetate, and then ethanol respectively, in the same manner as hexane. The solvent from different filtrates was removed under vacuum using a rotary evaporator at 40°C. The fractionated extracts of hexane (SE-HX), ethyl acetate (SE-EA), and ethanol (SE-EN) were kept in closed containers at 4°C for further studies.

The standardization of the obtained extracts was performed using high performance liquid chromatography (HPLC). The HPLC system was Prominence-i LC-2030 (Shimadzu, Kyoto, Japan) with UV detector, connecting with a reversed-phase C18 column, 4 mm i.d. × 250 mm (Eurospher II, Knauer, Berlin, Germany). The extract was dissolved in methanol to obtain a concentration of 1 mg/mL. The extract solution was filtered through a 0.22 µm filter membrane prior to injecting to the HPLC with an injected volume of 5 µL. The HPLC mobile phase consisted of 1% acetic acid and methanol (20:80 by volume) and was used at a flow rate of 0.6 mL/min with an isocratic condition for 45 min. Detection was performed by means of a UV detector at a wavelength of 280 and 360 nm. Sophoraflavanone G was reported to have strong anticancer activity against various cancer cells (*19,20*). In this study, sophoraflavanone G purified from *S. exigua* extract was used as a marker. The purification of sophoraflavanone G was done using silica gel column chromatography as described in a previous report (*21*).

2.3. Evaluation of antioxidant activity

This experiment was performed using a free radical scavenging method and ferric reducing antioxidant power (FRAP) assay previously described (*22,23*). In the radical scavenging method, the free radicals of ABTS were generated by mixing 8 mL of ABTS solution with 12 mL of 2.45 mM potassium persulfate solution. The resulting mixture was incubated in the dark for 16 h at room temperature. After that, ethanol was added until the absorbance of the mixture at 750 nm was approximately 0.7. Each extract sample used in this study was 0.5 mg/mL solution in ethanol. For liposome samples, the developed liposome formulations were added with Milli-Q water to obtain 50-fold dilutions. An aliquot of 20 µL of the sample solution was mixed with 180 µL of the ABTS free radical

solution, then incubated in the dark at room temperature for 5 min prior to measuring the absorbance at 750 nm using a microtiter plate reader (Bio-Rad, Model 680, Hercules, CA, USA). Trolox was used for calibration. In the FRAP assay, the FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM hydrochloric acid with 2.5 mL of ferric chloride and 25 mL of 0.3 M acetate buffer, pH 3.6. The amount of 20 µL of each sample solution was mixed with 180 µL of FRAP reagent in 96 well plate. The negative control or blank was prepared in the same manner without adding the sample. The samples and blanks were incubated for 10 min at room temperature prior to determining the absorbance at 595 nm using microplate reader (Bio-Rad, Model 680, Hercules). The reducing power of the samples was evaluated by calculating the amount of Fe^{2+} produced by the samples using the calibration curve of ferrous sulfate. BHT at 1 mg/mL in ethanol was used as a positive control. All experiments were run in triplicate.

2.4. Solubility and miscibility studies

SE-EA was used in this experiment. The solubility of SE-EA in various pharmaceutical solvents was investigated. The exact weight of 1 g SE-EA was dropped with a small amount of each test solvent. The mixture was mixed using a Vortex mixer. All mixtures were observed visually. Each solvent was added, and the mixture was mixed in the same manner until a clear solution was obtained. Each sample was performed in triplicate. The smallest amount of the solvent used to completely dissolve SE-EA was recorded. The approximate solubility of SE-EA in each solvent was expressed as amount of the solvent (mL) used to dissolved 1 g of SE-EA. For the miscibility test, each pharmaceutical excipient was mixed with SE-EA in a 1:1 weight ratio. The outer appearance of the obtained mixtures was visually inspected for compatibility.

2.5. Preparation of liposomes

In this study, SE-EA was used to prepare the liposomes. The preparation of SE-EA liposomes was performed using a thin film hydration method previously described with some modification (*24*). Probe sonication was used to assist the formation of desirable liposomes. The weight ratio of phosphatidylcholine and cholesterol was 9:1. Different liposomes were formulated as shown in Table 1, T1-T4 were those containing SE-EA and B1-B4 were the respective empty liposomes. For T1-T4, the liposomes composed of 1.0, 1.5, 2.0, and 2.5% of SE-EA and 4, 6, 8, and 10% of total lipid, respectively were prepared to obtain SE-EA:total lipid ratio of 1:4. For preparation of 100 mL SE-EA liposome dispersions, phosphatidylcholine and cholesterol were dissolved in 20 mL of chloroform-methanol mixture (volume ratio of 3:1) in a round bottom flask. After that, 1 mL of SE-EA in chloroform solution was added and mixed well. The obtained mixture was subjected to a vacuum rotary evaporator (N-1000, Eyela, Tokyo, Japan) at 40°C for 25 min to remove the solvents. The formed thin film layer was flushed under a stream of nitrogen for 1 min prior to re-suspending in Milli-Q water. The obtained dispersion was sonicated at 45°C using a Probe ultrasonicator (VCX 600, Sonics & Materials, CT, USA) at 50% amplitude pulse mode of 10,000 J by vibrating for 1 sec and stopping for 1 sec, for a total of 30 min. Pentylene glycol was used as a preservative of the system. Empty liposomes for each SE-EA liposome formulation were fabricated in the same manner without adding SE-EA. The obtained liposome dispersions were kept at 4°C and protected from light, prior to use.

Table 1. Compositions of SE-EA liposome and the empty liposome formulations

Formulations	SE-EA (%)	Total lipids (%)
SE-EA liposomes		
T1	1.0	4
T2	1.5	6
T3	2.0	8
T4	2.5	10
Empty liposomes		
B1	0	4
B2	0	6
B3	0	8
B4	0	10

2.6. Physicochemical properties of liposomes

2.6.1. Morphology and particle analysis

Morphology of the liposome particles in the dispersions was investigated by transmission electron microscopy (TEM) using a Hitachi HT 7800 electron microscope operating at 80 kV. The TEM samples were prepared by dropping 50-fold diluted liposome dispersion with Milli-Q water onto a 400-mesh copper grid coated with carbon film, and then negative staining with 0.2% (w/w) phosphotungstic acid. After that, the samples were dried in a vacuum desiccator at 25°C for 24 h.

The particle size, size distribution, and zeta potential of the liposomes in the dispersions were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano ZS (Malvern instrument, Worcestershire, UK) at 25ºC. Each sample was diluted 100-fold with Milli-Q water and subjected to sonication for 30 min before measurement. The hydrodynamic size and size distribution of the liposomes were measured at a fixed angle of 173. The particle size was expressed as the average diameter in nm, whereas the particle size distribution was expressed as the polydispersity index (PdI). The zeta potential of the samples was analyzed and automatically calculated based on the

Smoluchowski equation (*25*) using the Zetasizer (Malvern Instruments Company) software version 7.1. All experiments were performed in triplicate.

2.6.2. Crystalline characteristics

Prior to these studies, the liposome dispersions were lyophilized using a freeze dryer (Christ Beta 2-8 LD Plus, Christ Beta, Osterode am Harz, Germany). The obtained lyophilized powder samples were dried in a vacuum oven at 25°C for 24 h before use. Crystalline characteristics of the samples were investigated using an X-ray diffractometer (XRD) (Rigaku SmartLab, Rigaku, Tokyo, Japan) with Cu-Kα radiation at a voltage of 45 kV and 30 mA. A Bragg angle (2-theta) was used at a range of 10° to 60° with a step size of 0.01.

2.7. Determination of entrapment efficiency

The entrapment efficiency of SE-EA liposomes was determined using an indirect method. First, the total amount of SE-EA in the liposomes was determined. The liposomes were diluted 20 folds with methanol. The obtained solution was mixed at a volume ratio of 1:1 with 10% Triton X-100 to destroy the liposome structure. The resulting mixture was then diluted 20 folds with methanol, then subjected to a centrifuge (MPW-352R, MPW Med. instruments, Warsaw, Poland) at 12,000 rpm for 10 min at 4°C. After centrifugation, the supernatant containing the total amount of SE-EA from both inside and outside of the liposomes was determined at 280 nm using a UV spectrophotometer (UV-2600i, Shimadzu, Kyoto, Japan). Methanol was used as a blank. For determining the un-entrapped SE-EA, the liposome dispersion was subjected to a centrifuge (MPW-352R, MPW Med. instruments) at 15,000 rpm for 20 min at 4°C. The supernatant was diluted 200 folds with methanol prior to quantifying SE-EA using a UV spectrophotometer (UV-2600i, Shimadzu) at 280 nm.

A standard curve of sophoraflavanone G was constructed in a linear concentration interval ranging from 0.003 to 0.05 mg/mL. The amount of SE-EA was calculated using the following equation: EE (%) = (Ao – Af) × 100/Ao. Where, Ao represents the total amount of SE-EA in the formulation. Af represents the amount of the free SE-EA in the supernatant.

2.8. *In vitro* release study

The release profile of SE extract from each liposome was determined by a dialysis method using a dialysis bag (regenerated cellulose tubular membrane with MWCO of 12,000; Cellu Sep T1, Siguin, TX, USA). A phosphate buffered solution (PBS) of pH 7.4 containing 30% (v/v) ethanol and 2% (v/v) Tween 80 was used as a release medium. An exact volume of 1 mL of SE-EA liposome dispersion was placed in pre-swollen dialysis bags. The dialysis bags were transferred into 500 mL of the release medium with stirring at 100 rpm at 37°C using a magnetic stirrer (Rexim RSH-4DR, As One, Osaka, Japan). An aliquot of 5 mL of the release medium was withdrawn and the fresh medium with the same volume was replaced at time intervals of 2, 4, 7, 10, 15, 24, 36, 48, and 72 h. The amount of the released SE-EA was measured using UV spectrophotometer (UV-2600i, Shimadzu) at 280 nm. The percentage of SE-EA cumulative release was determined. All experiments were performed in triplicate.

2.9. Statistical analysis

All experiments were performed in triplicate. Data of particle size, size distribution, and zeta potential as well as entrapment efficiency and release property are expressed as mean ± SD. The obtained data were analyzed statistically by SPSS statistic 22.0 software. The mean of each test was determined for significance at $P < 0.05$ by ANOVA and Tukey's Multiple tests.

3. Results

3.1. Preparation and standardization of SE-EA

The outer appearance of *S. exigua* root extracts from hexane, ethyl acetate, and ethanol were different. SE-HX appeared as a yellowish-brown viscous liquid and SE-EA was an orange-brown semisolid whereas SE-EN presented as a yellowish-brown semisolid. Among them, SE-EA significantly showed the highest yield of 9.2 ± 0.5%, followed by SE-EN and SE-HX which showed the yields of 7.9 ± 0.9% and 4.7 ± 0.8%, respectively. The outer appearance of sophoraflavanone G was yellowish-orange powder. This compound showed a maximum absorption at 280 nm as shown in Figure 1. HPLC chromatogram of sophoraflavanone G at 280 nm showed identical absorption peak at 11.02 min as

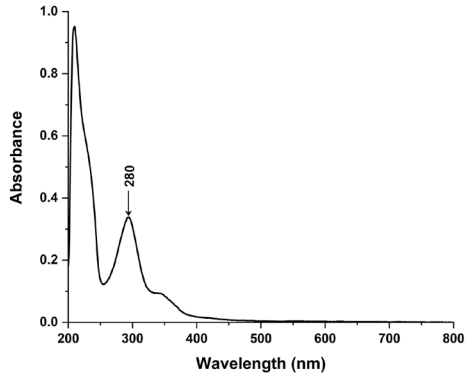

Figure 1. UV spectrum of sophoraflavanone G.

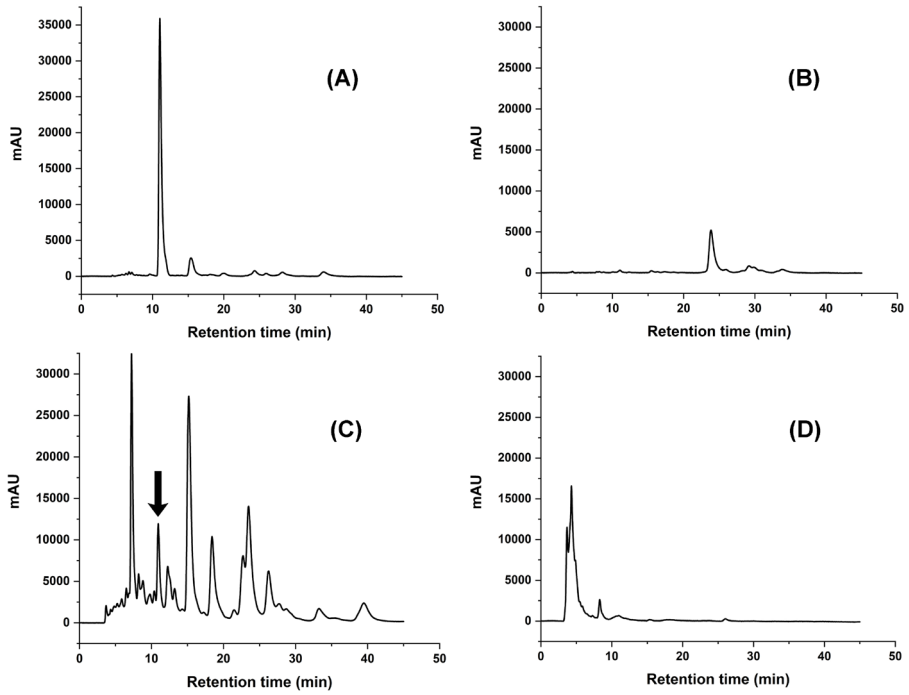

Figure 2. HPLC chromatogram of sophoraflavanone G (A), SE-HX (B), SE-EA (C), and SE-EN (D).

shown in Figure 2A. HPLC chromatogram at 280 nm of the three extracts showed different characteristics as seen in Figures 2B-2D for SE-HX, SE-EA, and SE-EN, respectively. The results obviously demonstrated that only SE-EA chromatogram showed an identical peak at the same retention time as sophoraflavanone G (indicated by the black arrow), which confirmed that SE-EA contained sophoraflavanone G. The HPLC chromatograms of SE-HX and SE-EN showed no sophoraflavanone G peak.

3.2. Antioxidant activity of the extracts

The three fractionated extracts of *S. exigua* were subjected to two standard methods of antioxidant test for free radical scavenging activity and reducing capacity. The free radical scavenging activity of each sample was expressed as Trolox equivalent antioxidant capacity or TEAC. This index is defined as the millimolar concentration of a Trolox solution whose antioxidant capacity is equivalent to 1.0 mg of the extract. In the investigation of reducing property of the extracts, Fe^{2+} concentrations from ferrous sulfate solutions were used as a calibration curve. The reducing power of the extracts was expressed as equivalent concentration or EC. This parameter was defined as the concentration of the extract having a ferric reducing property equivalent to that of 1 mM of ferrous sulfate. The results showed that SE-EA possessed the highest property of both radical scavenging activity and reducing capacity followed by SE-HX and SE-EN, respectively as shown in Table 2.

3.3. Solubility and miscibility of SE-EA

Table 2. Antioxidant activity of the three fractionated extracts

Extracts of *S. exigua*	TEAC* (mM/mg extract)	EC* (mM/mg extract)
SE-HX	16.56 ± 3.11b	38.45 ± 3.74b
SE-EA	67.31 ± 0.66a	67.23 ± 2.08a
SE-EN	17.95 ± 0.58b	39.05 ± 3.60b

Lowercase letters indicate significant difference between treatment groups ($P < 0.05$).

Table 3. Solubility of SE-EA in certain pharmaceutical solvents

Solvent	Solubility (mg/mL)
Deionized water	< 1
Methanol	12.5
Ethanol	25
Chloroform	50
Dimethyl sulfoxide	50
Acetone	50
Dichloromethane	< 1
Diethyl ether	1.25

Solubility of SE-EA in several kinds of pharmaceutical solvents commonly used for liposome preparation is shown in Table 3. It was found that the solubility of SE-EA was very low (less than 1 mg/mL) in a highly polar solvent like water. It was obviously observed that SE-EA solubility increased when less polar solvents than water such as methanol, ethanol, chloroform, dimethyl sulfoxide, and acetone were used. However, the solubility of SE-EA dramatically decreased when highly non-polar solvents such as dichloromethane and diethyl

ether were used. Therefore, water, dichloromethane, and diethyl ether are not suitable solvents for SE-EA. Among the moderate polar solvents, SE-EA showed the highest solubility in chloroform, dimethyl sulfoxide, and acetone, followed by ethanol and methanol, respectively. Pharmaceutical excipients such as surfactants and polyethylene glycols (PEG) are always used in pharmaceutical preparations. SE-EA showed different miscibility to different kinds of excipients as shown in Figure 3. It was found that SE-EA was immiscible with an oil-soluble surfactant like Span 80 but miscible with a water-soluble surfactant such as Tween 80 and Triton X-100. In addition, the extract showed well miscible with PEG 200 and PEG 400.

3.4. Morphology and particle analysis

The shape of SE-EA liposomes and the empty liposomes was observed using TEM. The results are illustrated in Figure 4. All liposomes appeared as a spherical shape. In addition, the TEM images revealed that the approximate size of SE-EA liposomes were slightly bigger than their respective empty liposomes. Particle analysis using PCS could determine the exact particle size, size distribution, and zeta potential of the liposomes. The results are shown in Table 4. It was found that the mean size of SE-EA liposomes was in the range of 166.87-245.77 nm, significantly bigger than that of their respective empty liposomes which was in the range of 94.81-119.23 nm. The size distribution of SE-EA liposomes was also wider (0.395-0.474) than their empty liposomes (0.151-0.218). In addition, the zeta potential of SE-EA liposome was more negative value than their empty liposomes. The difference in size, size distribution, and zeta potential of SE-EA liposomes was dependent on the SE-EA and total lipid concentrations.

3.5. Crystalline characteristics

The results of this study revealed XRD diffractograms of the test materials as shown in Figure 5. It was found that the internal structure of both cholesterol and phospholipids was crystalline structure. Cholesterol showed major identical peaks at 2θ of 10.6, 12.9, 15.5, 16.9, 20.7, and 23.4° with one strong peak at 15.5° while phospholipids exhibited one major crystalline

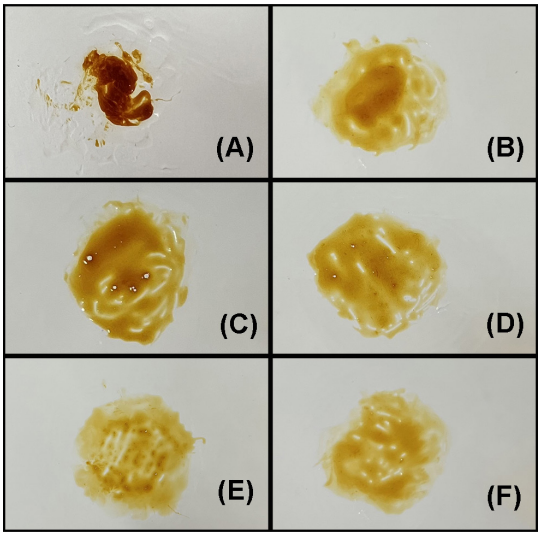

Figure 3. Outer appearance of the mixtures showing the miscibility of SE-EA and Span 80 (A), Tween 80 (B), Tween 20 (C), Triton X-100 (D), PEG 200 (E), and PEG 400 (F).

Table 4. Mean size, PdI, and zeta potential of the liposomes

Formulations	Size (nm)	PdI	Zeta potential (mV)
T1	172.97 ± 5.67	0.402 ± 0.024	–34.43 ± 0.21
T2	184.97 ± 6.34	0.474 ± 0.010	–37.70 ± 0.53
T3	199.87 ± 0.68	0.413 ± 0.029	–36.50 ± 0.46
T4	245.77 ± 22.19	0.395 ± 0.002	–44.47 ± 0.90
B1	94.81 ± 2.78	0.206 ± 0.020	–23.23 ± 0.55
B2	103.10 ± 1.80	0.207 ± 0.006	–27.40 ± 0.92
B3	111.50 ± 0.46	0.218 ± 0.043	–27.97 ± 0.91
B4	119.23 ± 1.97	0.151 ± 0.008	–32.03 ± 1.26

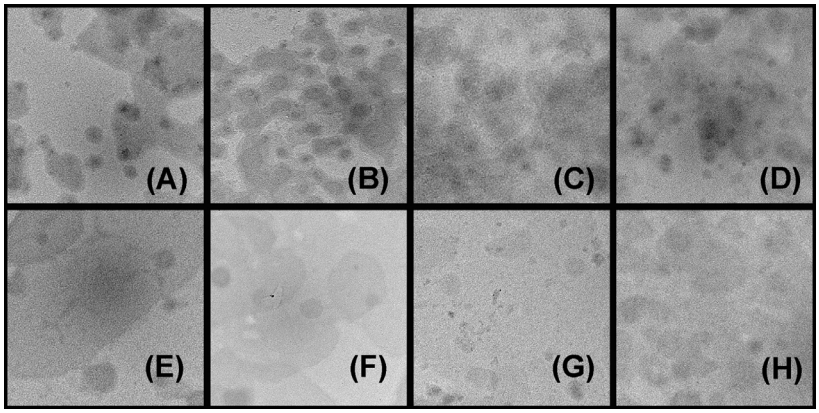

Figure 4. TEM images of the prepared liposomes: T1-T4 (A-D) and B1-B4 (E-H).

peak at 26.4°. The XRD of SE-EA demonstrated a halo pattern indicating that the internal structure of this extract was amorphous form. Interestingly, all four SE-EA liposomes (T1-T4) and their respective empty liposomes (B1-B4) showed only one crystalline peak at about 21.4-21.5°. This result indicated that the crystalline structure of cholesterol was disrupted and changed to the amorphous form.

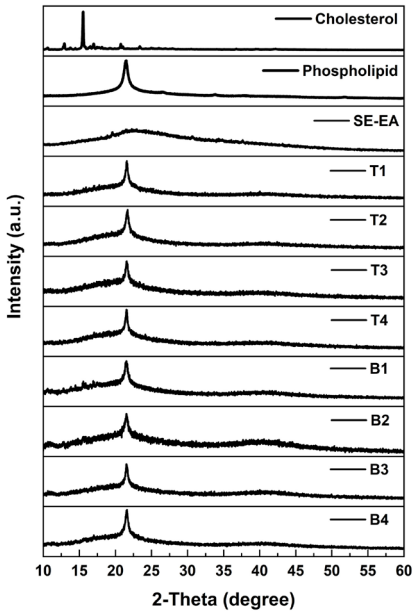

Figure 5. XRD of the liposomes in comparison with their intact lipids and SE-EA.

3.6. Investigation of entrapment efficiency and the release property of SE-EA liposomes

The results of this study indicated that the efficiency of the four SE-EA liposome formulations on entrapment of SE-EA were different. Among them, the entrapment efficiency of T4 was the highest (65.32 ± 0.07%), followed by that of T3 (50.31 ± 0.01%). The EE values of T1 and T2 were similar with the values of 47.47 ± 0.10% and 47.63 ± 0.21%, respectively.

Study on release property of SE-EA liposomes for 3 days indicated that each formulation could release SE-EA, but quite different behavior as seen in Figure 6. T1 showed the fastest release property followed by T2 and T3, respectively. The slowest release property was observed in T4. Within 15 h, more than 50% of SE-EA was released from T1, whereas less than 50% release was found in the other formulations. In addition, T3 and T4 exhibited significant time lags in releasing SE-EA, respectively. At the end of the test period (72 h), the maximum cumulative release of T1 was 88.27 ± 1.96%, whereas that of T2, T3, and T4 were 82.41 ± 2.94%, 74.95 ± 3.64%, and 65.66 ± 2.95%, respectively.

3.7. Antioxidant activity of SE-EA liposomes

The antioxidant activity of the developed SE-EA

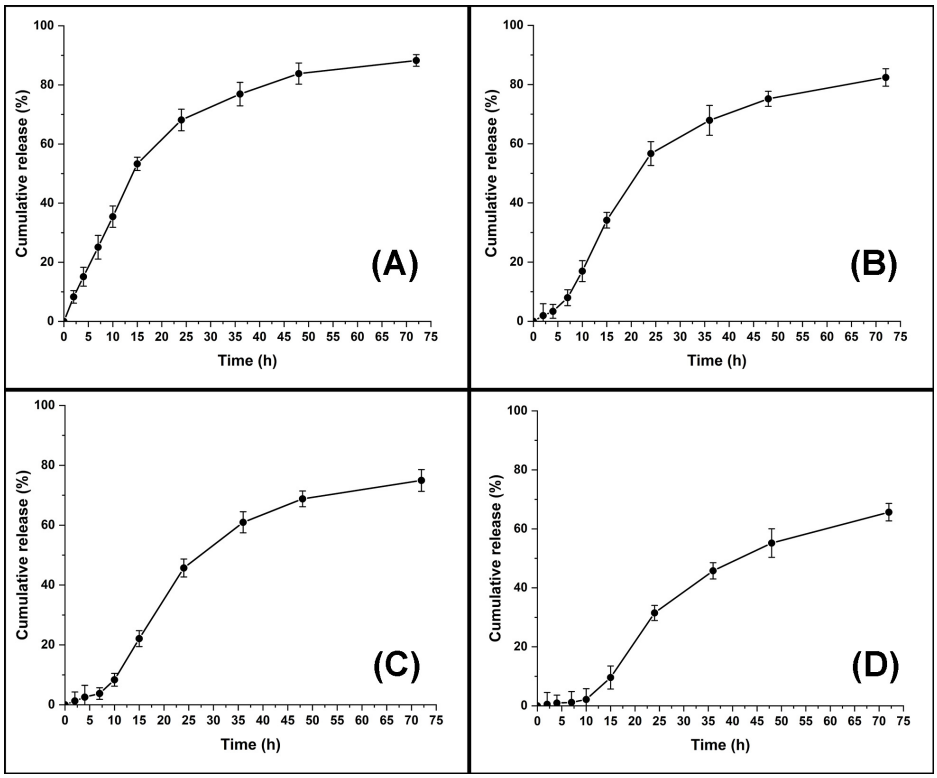

Figure 6. Release property of SE-EA liposome formulations: T1 (A), T2 (B), T3 (C), and T4 (D).

liposome formulations was investigated for free radical scavenging activity and reducing capacity by using ABTS and FRAP assays, respectively. It was found that T4 exhibited the highest free radical scavenging activity with the TEAC value of 346.45 ± 2.71 mM/mg, followed by T3, T2, and T1 with the TEAC values of 186.54 ± 2.12, 146.92 ± 7.15, and 117.54 ± 2.75 mM/mg, respectively. The results from FRAP indicated that T4 possessed the highest reducing power with the EC value of 84.16 ± 5.01 mM/mg, followed by T3, T2, and T1 with the EC values of 73.65 ± 4.41, 62.89 ± 3.74, and 48.13 ± 5.63 mM/mg.

4. Discussion

Our findings indicate that SE-EA showed the strongest free radical scavenger among the three fractionated extracts of *S. exigua*. It shows ABTS free radical scavenging capacity with the TEAC and EC values of 67.31 ± 0.66 and 28.32 ± 1.83 mM/mg extract, respectively. Therefore, SE-EA can be considered as one of the important sources for natural antioxidants. The active components of *S. exigua* extracts are flavonols, flavones, chromones, and pterocarpans. It has been reported that the phenolic compounds in the root of *S. exigua* were identified as exiguaflavanones A–M and a benzochromone, exiguachromone B (*26–28*). Sophoraflavanone G was reported to have strong anticancer activity against various cancer cells (*19,20*). This active compound has been reported to have antioxidant activity that can suppress oxidative stress in an animal murine asthma model (*29*). In the present study, extraction of *S. exigua* root using hexane, ethyl acetate, and ethanol, respectively can yield different kinds of extracts, SE-HX, SE-EA, and SE-EN, respectively. Among these three extracts, SE-EA has the highest content of sophoraflavanone G. This result indicates that sophoraflavanone G is one of the active components that contribute to antioxidant activity of SE-EA. The solubility result shows that SE-EA cannot dissolve in the highly hydrophilic solvent like water or highly lipophilic solvent like diethyl ether, but it can dissolve well in the moderate polar solvents like chloroform, dimethyl sulfoxide, acetone, ethanol, and methanol, respectively. Pharmaceutical formulations of SE-EA with these organic solvents may cause irritation and some severe side effects due to the solvents (*30,31*). Furthermore, due to the nature of sophoraflavanone G and some other active compounds in SE-EA, they may not be able to penetrate cell membrane to reach the required bioavailability and show antioxidant efficacy. Liposomes possess several unique properties that can enhance solubility, bioavailability, and cellular uptake as well as stability of several drugs. Several studies are being carried out to deliver liposomes to various tissues, *e.g.*, retina (*32*), lung (*33*), inner ear (*34*), and vagina (*35*). In addition, there are several routes which are available for administration of liposome such as oral, parenteral, and topical. Several bioactive compounds from plants have been reported to be entrapped in liposomes (*36–38*). Therefore, SE-EA liposomes were developed to overcome the disadvantages of the extract. In the current study, the liposomes were prepared using a simple film method with the aid of sonication. Two types of sonication techniques, bath sonication and probe sonication can be applied for liposome preparations (*39*). The bath sonication is suitable for large amount liposome production. In the current study, a small scale of liposomes production was performed. Therefore, probe sonication was used. The obtained liposomes are spherical in shape with different sizes and entrapment efficiencies. All prepared liposomes exhibited negative zeta potential, suggesting the negative charge on their surface. It is considered that the negative zeta potential of the liposomes can be due to the phosphate groups of the phospholipids (*40*). Zeta potential is an indication of the physical stability of the liposomes. Liposomes are considered stable when their zeta potential is higher than 30 mV, regardless of charge (*41*). It is found that SE-EA liposomes are slightly bigger than their respectively empty liposomes. It is considered that the enlargement of SE-EA liposome is due to the intercalation of the extract inside the layer of the liposomes.

The result of XRD indicates that the internal structure of the obtained liposomes has crystalline characteristics of only phospholipids but no crystalline structure of cholesterol. It is considered that cholesterol and the extract may miscible together to yield a solid solution or amorphous form. The entrapment efficiency and the release property of the liposomes depends on the total amount of the lipid components. It is found that high lipid concentration yields the liposomes with high entrapment efficiency but slow-release behavior. Our study shows that SE-EA liposome formulation T1 which is composed of 4% total lipid concentration has the lowest entrapment efficiency of 47.47 ± 0.10% whereas the liposome T4 formulation which is composed of 10% total lipid content show the highest entrapment efficiency of 65.32 ± 0.07%. Low amount of total lipid content causes fast release whereas high amount of lipid content retards the release ability of the liposomes. The results of our study can be concluded that SE-EA liposomes can be successfully prepared using thin film hydration methods. T1 is the most suitable liposome formulation for fast release and T4 is the best formulation for sustained release behavior. All SE-EA liposomes show significantly stronger free radical scavenging and reducing properties than the non-entrapped extract. This result is in good agreement with the previous study that the myrtle berry extract loaded liposomes exhibited higher free radical scavenging activity than the extract solution (*42*). It has been reported that empty liposomes contain only phosphatidylcholine or a mixture of phosphatidylcholine and cholesterol possess antioxidant activities with high TEAC and EC values (*43,44*). In

the present study, our developed SE-EA liposomes are composed of both phosphatidylcholine and cholesterol, therefore, the liposomes showed significantly higher antioxidant activity than the non-entrapped SE-EA.

Acknowledgements

The authors would like to thank the Center of Excellence in Pharmaceutical Nanotechnology, Chiang Mai University for supporting facility and equipment.

Funding: This research was supported by Fundamental Fund 2023, Chiang Mai University and Thailand Science Research and Innovation, Thailand (Grant number 180072_FF66/046).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received March 19, 2024; Revised May 4, 2024; Accepted May 14, 2024.

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Released online in J-STAGE as advance publication May 22, 2024.