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Development and assessment of tyrosinase inhibitory activity of liposomes of *Asparagus racemosus* extracts

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ABSTRACT

The purpose of this study was to develop liposomal formulations of *Asparagus racemosus* root extract (AR1-6) as well as evaluate the physicochemical properties and *in vitro* tyrosinase inhibitory activity. Liposomes composed of AR1-6 to lipid weight ratio of 1:10 and lecithin (LEC) or Phospholipon® 90G (PC90G) as structural phospholipid at 7:3 molar ratio to CHOL were prepared by various methods, i.e. chloroform-film (CF), reverse-phase evaporation (REV), polyol dilution (PD), and freeze-drying of monophase solution (MFD) methods. The results revealed that vesicles prepared by CF and MFD were multilamellar whereas those prepared by REV and PD were oligolamellar in nature with particle sizes ranging from 0.26 to 13.83 μm. The zeta potentials were in the range of −1.5 to −39.3 mV. AR1-6 liposomes with LEC possessed significantly higher entrapment than those with PC90G. The highest entrapment efficiency and *in vitro* tyrosinase inhibitory activity of 69.08% and 25%, respectively, were obtained from liposomes having LEC and prepared by PD method. The tyrosinase inhibitory activity were in the rank order of LEC > PC90G, and PD > CF > REV > MFD. It could be concluded that the mechanism of vesicle forming in each method of preparation was the key factor influencing physicochemical properties, particularly vesicle type, size, surface charge, and entrapment, which were well correlated with the biological activity.

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1. Introduction

The increase in production and accumulation of melanin are the cause of a large number of skin ailments, e.g. acquired hyperpigmentation such as melisma, postinflammatory melanoderma, solar lentigo, etc. The hyperpigmentation of the epidermis and dermis depend on either increased numbers of melanocytes or the activity of the enzyme [1]. The melanocytes are responsible for the biosynthesis of melanin through enzymatic conversion of L-tyrosine which leads to the suntanning effect. However, overproduction of melanin pigment is not desirable, especially on people's face [2,3].

The plant *Asparagus racemosus* Willd. (Liliaceae) is commonly called, in India, Satavari; Satawar or Satmuli in Hindi; Satavari in Sanskrit; Shatamuli in Bengali, etc. In Thailand it is called Sam-Sib or Sam-Roi-Rak. The plant is a spinous under-shrub, with tuberous, short rootstock bearing numerous succulent tuberous roots (30–100 cm long and 1–2 cm thick), which are silvery white or ash colored externally and white internally [4]. Pharmacological studies with animals have manifested the potency of *A. racemosus* extract as an antioxidant, anticancer, anti-inflammatory, anti-aging, and as a substrate of inulinase production for ability in modulating the immune system [5]. Moreover, the tyrosinase inhibitory activity as skin whitening effect has also been found from the investigation of this extract by using dopachrome microplate assay [6,7]. The root extracts of *A. racemosus* have been employed in two major forms as methanolic and aqueous extracts, the products of which were in the form of tablets and syrup [4]. It has been reported that the major active constituents of *A. racemosus* are steroidal saponins (Shatavarins I–IV) present in the roots [4]. Besides, other active compounds such as quercetin, rutin, kaempferol, racemofuran, and isoflavone were also found and identified on the basis of chemical and spectroscopic evidence. It has previously been observed that a number of flavonoids were found to manifest the tyrosinase inhibitory activity with some flavonols acting as copper chelators [29]. Besides, quercetin and kaempferol were found to inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase. As a result, *A. racemosus* extract might be a candidate for therapeutic applications in preventing pigmentation disorders and other melanin-related health problems as well as for cosmetic applications for skin whitening effect.

Liposomes have long been receiving a lot of attention during the past thirty years as drug targeting systems of great potential. More recently, many new developments have been emerging in the area of liposomal drugs, from clinically approved products to new experimental applications, with gene delivery and cancer therapy still being the principal areas of interest [8]. The pharmaceutical and pharmacological justifications of the use of liposomes as drug carriers are well recognized [9]. Numerous liposomal drugs have been approved for undergoing clinical evaluation, e.g. cytarabine, vincristine, lurtotecan, platinum compounds, DNA plasmid encoding HLA-B7 and α2 microglobulin, all-*trans* retinoic acid, and *E1A* gene [8].

In this study, liposomes of *A. racemosus* extract for therapeutic applications in preventing pigmentation disorders were prepared by various methods, i.e. chloroform-film, reverse-phase evaporation, polyol dilution, and freeze-drying of monophase solutions methods, their physicochemical properties and tyrosinase inhibitory activities of which were evaluated.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC90G) (Phospholipon® 90G) was a gift from Rhône Poulenc Rorer, Köln, Germany. Lecithin (LEC) was purchased from Union Chemicals, Bangkok, Thailand. Cholesterol (CHOL) and *tert*-butanol (TBN) were obtained from Carlo Erba Reagents, Rodano, Italy. Diosgenin (DG) was obtained from Sigma–Aldrich®, St. Louis, MO, USA. Kojic acid, 3,4-dihydroxy-L-phenylalanine (L-DOPA), mushroom tyrosinase, sucrose (SUC), and vanillin were purchased from Sigma–Aldrich®, Steinheim, Germany. Chloroform, methanol, and sulfuric acid, 96% (v/v), were from Labscan, Stillorgan, Co Dublin, Ireland. Dibasic potassium phosphate, monobasic potassium phosphate, and sodium hydroxide were from Ajax® Finechem, Auckland, New Zealand. Absolute ethanol was from Merck KGaA, Darmstadt, Germany.

2.2. Plant materials

The roots of *A. racemosus* were collected from Tak Province, Thailand. The plants were identified by the botanical staff and the voucher specimen of the plant was deposited in the herbarium of Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand.

2.3. Extraction of A. racemosus roots (AR1-6)

A. racemosus roots were dried and minced into powder and then extracted by maceration with 95% ethanol at 25 °C for 24 h. The supernatant was filtrated and evaporated by a rotary evaporator (Eyela® A-3S, Tokyo Rikakikai, Japan). The dark brown viscous liquid obtained thereafter was freeze-dried (Christ® LOC-1m, Göttingen, Germany), and kept at 4 °C until use.

2.4. Thin layer chromatography (TLC) fingerprint

TLC was performed on silica gel plate, $60F_{254}$ (Merck KG, Darmstadt, Germany) and the developing system of 2:1-hexane:ethyl acetate was used in this study [10]. Solution of DG (0.5 mg/ml) in methanol was used as reference [11,12]. The sample solution of AR1-6 (10 mg/ml) was prepared by dissolving in methanol and then filtering through 0.2-μm nylon membrane filter before use. DG solution and AR1-6 sample at 8 μl were spotted by Autospot equipped with scanner and camera (Camag®Limonat V, Switzerland) on the TLC plate, which were then air-dried for 5 min and run with the developing system. After running was complete, the plate was dried in the oven at 100–110 °C for 5 min, sprayed with 10 ml anisaldehyde-sulfuric acid reagent, and then heated at 105 °C for 5–10 min. Afterward, the plate was visualized under visible (366 nm) and UV light (254 nm) (Perkin Elmer® Lambda 35, Waltham, Massachusetts, USA) and TLC fingerprints were photographed. This experiment was performed in duplicate.

2.5. Total saponins quantification

Total saponin content of AR1-6 was determined as previously described [11,13]. Sample solutions of DG (0.5 mg/ml) and AR1-6 (5 mg/ml) in methanol were prepared and filtered through 0.2-μm nylon membrane filter before use. A 250-μl portion of 8% w/v vanillin solution in absolute ethanol was added to 250 μl of sample, which was subsequently mixed with 2.5 ml of 72% v/v sulfuric acid, then incubated in water bath at 60 °C for 10 min, and finally cooled in ice bath for 5 min. The absorbance of this mixture was measured at 544 nm (Perkin Elmer® Lambda 35, Waltham, Massachusetts, USA). The calibration curve of DG at a high degree of linearity with correlation coefficient (r^2) of 0.9998 was achieved across the specified range of 16.7–83.3 μg/ml. The calibration curve of AR1-6 at a high degree of linearity with correlation coefficient (r^2) of 0.999 was achieved across the specified range of 1.7–416.7 μg/ml. The diosgenin equivalent (DGE) (μg/mg extract) was used as reference in determining the total amount of saponins in AR1-6. This experiment was performed in triplicate.

2.6. Preparation of AR1-6 liposomes by various methods

2.6.1. Chloroform-film method (CF)

Liposomes containing AR1-6 to lipid weight ratio of 1:10 and phospholipid (LEC or PC90G) to CHOL molar ratio of 7:3 were prepared by chloroform film method. Total lipid mixture of 100 mg was dissolved in 20 ml chloroform in a 100-ml round bottom flask. An accurate amount of 10 mg AR1-6 was dissolved in 10 ml methanol, which was subsequently added to the lipid mixture solution and mixed until homogeneous solution was obtained. The rotary evaporator (Eyela® A-3S, Tokyo Rikakikai, Japan) was used to evaporate out the organic solvent at 35 °C for 1 h. After a dry thin film was obtained on the bottom of flask, phosphate buffer saline (PBS) pH 7.0 was added to hydrate the lipid film and mixed continuously at 60 °C for 1 min. The dispersion was then completely hydrated at 54 °C for 2 h and subsequently sonicated in an ultrasonic bath (Branson® Model B-12, Branson Cleaning Equipment, USA) at 25 °C for 30 min.

The unentrapped free drug and lipids were separated by ultracentrifugation (Optima® L-80 XP, Fullerton, California, USA) at 20,000 rpm for 20 min, after which the precipitates were resuspended and washed several times with PBS pH 7.0 until concentration of drug in the supernatant was less than 2.0 μg/ml. The purified liposomes were finally filled and sealed in 20-ml vial and kept at 4 °C prior to use.

2.6.2. Reverse-phase evaporation method (REV)

Liposomes containing AR1-6 to lipid weight ratio of 1:10 and phospholipid (LEC or PC90G) to CHOL molar ratio of 7:3 were prepared by reverse-phase evaporation method as previously described [14]. Total 100 mg mixture of all compositions was dissolved in 60 ml chloroform, after which 20 ml of PBS pH 7.0 was added. Subsequently, the dispersion was sonicated using an ultrasonic bath (Branson® Model B-12, Branson Cleaning Equipment, USA) at 7 °C for 10 min to produce water-in-oil (w/o) emulsion. The emulsion was then transferred to a rotary evaporator (Eyela® A-3S, Tokyo Rikakikai, Japan) to slowly evaporate the solvent out under vacuum at 35 °C until the 'viscous gel' was visualized, indicating the phase reversion stage into oil-in-water (O/W) emulsion. Continuation of evaporation would finally result in the formation of bilayer structure of liposomes, which could be characterized by a uniformly aqueous dispersion. The evaporation process was still progressing at a slightly higher temperature for a period of time to remove the organic trace as much as possible. Eventually, these liposomal dispersions were adjusted to initial volume by PBS pH 7.0. The obtained liposomal dispersions were subsequently purified as described above, and finally kept at 4 °C until use.

2.6.3. Polyol dilution method (PD)

Liposomes containing AR1-6 to lipid weight ratio of 1:10 and phospholipid (LEC or PC90G) to CHOL molar ratio of 7:3 were prepared by polyol dilution method with slight modification as previously described [15]. Total 100 mg mixture of all compositions were dissolved together in 4 ml of propylene glycol at 40 °C, which was subsequently injected into 16 ml of sterile water for injection at 40 °C with continuous stirring at 800 rpm for 15 min, and then cooled to room temperature. The obtained liposomal dispersions were subsequently purified as described above, and finally kept at 4 °C until use.

2.6.4. Freeze-drying of monophase solution method (MFD)

Dry reconstituted liposomal powder containing AR1-6 to lipid weight ratio of 1:10 and phospholipid (LEC or PC90G) to CHOL molar ratio of 7:3 were prepared by freeze-drying of monophase solutions with slight modification as previously described [16]. PC, CHOL, and AR1-6 were dissolved together in TBN and subsequently mixed with 25% w/v aqueous SUC solution at TBN to water ratio by weight of 3:2 to obtain a clear isotropic monophase solution, which was then filled into 5-ml freeze-drying vials. The vials were kept in the freezer at −80 °C for 8 h, after which the frozen samples were freeze-dried (Christ® LOC-1m, Göttingen, Germany) for 24 h at condenser temperature of −50 to −55 °C and pressure of 0.18 mbar. The dry reconstituted liposomal powders obtained were kept at 4 °C until use.

2.7. Physicochemical characterizations of prepared AR1-6 liposomes

2.7.1. Microscopic appearance

In order to examine the microscopic appearance of lamellarity of the prepared vesicles, negative staining transmission electron microscopy (TEM) (Jeol® JEM-2100, Tokyo, Japan) was performed. The procedures for staining the sample were as follows: a drop of liposomal suspension was placed on a 300-mesh copper grid. After waiting 5 min to allow the liposome to adsorb to the grid, the excess liquid was removed with the aid of filter paper. The sample remained on the grid was stained with 1% phosphotungstic acid and allowed to air dry for approximately 10 min, then osmium tetroxide (OsO_4) was used as a fixative in order for vesicle borders to be shown.

2.7.2. Particle size

The volume particle size and size distribution of liposomal dispersions were measured by laser diffraction using a Sympatec HELOS system (Mastersizer® 2000, Malvern

instrument, Worcestershire, UK) for measuring range of 0.02–2000 μm. All determinations were carried out in triplicate. The size distribution was characterized by the distribution parameters d10%, d50%, d90% and the distribution width expressed as 'span', which was calculated by the following equation:

$$\text{Span} = \frac{\text{d90\%} - \text{d10\%}}{\text{d50\%}} \quad (1)$$

2.7.3. Particle surface charge

The electrophoretic mobility and zeta potential of liposomal dispersions were determined by laser Doppler electrophoretic mobility measurements (Zetasizer® Nano ZS Series, Malvern instrument, Worcestershire, UK). The measurement was performed after dispersing the sample in PBS pH 7.0 at room temperature and filling about 1 ml of each liposomal dispersion into the zeta-potential cell. Briefly, the zeta-potential was detected by the movement of a charged surface with respect to an adjacent liquid phase and the mean value of zeta potential from three determinations was reported with standard deviation.

2.7.4. Entrapment efficiency

In order to determine the contents of AR1-6 encapsulated in liposomes, methanol was used to deliberate AR1-6 from liposomes. The total saponin content of AR1-6 entrapped in liposomes was determined as described above. All examinations were carried out in triplicate. The entrapment efficiency was calculated as follows:

$$\%\ \text{Entrapment efficiency} = \frac{\text{Amount of drug entrapped in liposomes}}{\text{Total amount of drug added in liposomes}} \times 100 \quad (2)$$

2.7.5. Tyrosinase inhibitory activity

The assay of enzyme inhibition activity of AR1-6 entrapped in liposomes was performed by using commercially available mushroom tyrosinase as previously described [17,18]. L-DOPA and kojic acid were also used as substrate and positive control in this experiment, respectively. The activity of tyrosinase inhibition was determined spectrophotometrically by monitoring dopachrome formation at 492 nm. The potential tyrosinase inhibitor will cause a decrease in dopachrome absorption. In brief, a 80 μl of 20 mM phosphate buffer (pH 6.8) was added to each well containing 40 μl of sample solution in a 96-well microtiter plate (Nunc®, Roskilde, Denmark), then followed by 40 μl of mushroom tyrosinase solution (427 units/ml). After each well was mixed and pre-incubated at 25 °C for 10 min, a 40 μl of 0.85 μM L-DOPA was added to the mixture, then mixed and incubated at 25 °C for 20 min. The absorbance of each well was then measured at 492 nm by using a microplate reader (Infinite® 200 PRO, Tecan Trading AG, Männedorf, Switzerland). All samples were run in triplicate. The inhibitory effects of the test samples were expressed as the percentage of tyrosinase inhibition as follows:

$$\%\ \text{Tyrosinase inhibition} = \left[\frac{(A-B)-(C-D)}{(A-B)}\right] \times 100 \quad (3)$$

where A and B denote the optical density at 492 nm of the mixture without test sample (L-DOPA mixed with enzyme in buffer; the control) and without test sample and enzyme (L-DOPA in buffer; the blank), respectively. C and D denote those with test sample and enzyme (L-DOPA mixed with enzyme and test sample in buffer; the reaction mixture) and without enzyme (L-DOPA mixed with test sample in buffer; the blank of C).

3. Results and discussion

3.1. Standardization of AR1-6

It has been reported that A. *racemosus* extracts contained saponins for the main constituents [19]. Until now, it has been found that the major constituents of A. *racemosus* in methanolic extract were steroidal saponins, which were separated, purified, and named as "Shatavarin". Ten derivatives of Shatavarin were numbered as Shatavarin I to X [4,5]. Gautam et al. [20] reported that A. *racemosus* aqueous decoction contained steroidal saponin, alkaloids, proteins, starch, tannin, and mucilage. Agrawal et al. [21] carried out screening tests and reported the positive results for steroids, phytosterols, carbohydrates, tannins, anthraquinones, saponins, glycosides and flavonoids, and negative results for terpenoids, amino acids and alkaloids. Most recently, Visavadiya et al. [19] have found that most of the phytoconstituents in A. *racemosus* root were saponins (8.833%), while the rest were polyphenols (1.692%), phytosterols (0.79%), ascorbic acid (0.762%), and flavonoids (0.476%).

Due to the fact that saponins were found in both aqueous and methanolic extracts of A. *racemosus* and could be purified successfully to pure compounds, they were considered as marker in this study. Standardization of the extract in this study was to confirm the presence of saponins and to control the quality of the AR1-6 by examining the TLC fingerprints and total saponin content.

3.2. Thin layer chromatographic (TLC) fingerprints

In this study DG was used as reference for steroid saponins. The TLC fingerprints of DG and AR1-6 run by using mobile phase system I (2:1—hexane:ethylacetate) are shown in Fig. 1, the R_f values and color zones of which are compiled in Table 1. The anisaldehyde-sulfuric acid reagent was used to detect phenol, sugar, steroid, and terpene that will turn violet, blue, red, grey or green. The results showed that DG and AR1-6 exhibited dark yellowish green and light blue color, respectively. These indicated that compounds detected in AR1-6 with this reagent may have the structure of phenol, sugar, steroid, and/or terpene in their molecules. This information

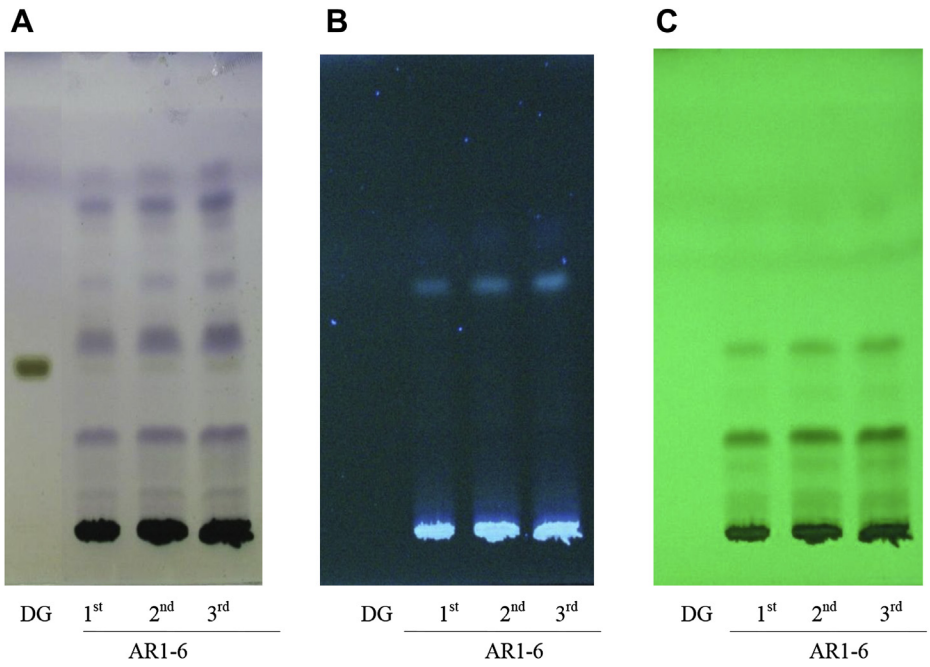

Fig. 1 – TLC fingerprints under visible light (A), UV long wavelength (366 nm) (B), and UV short wavelength (254 nm) (C) of AR1-6 sample run by using system I.

was useful for grouping of compounds such that TLC fingerprints of AR1-6 would be useful for identification of the extract and detection of alteration in the extract as well.

3.3. *Physicochemical characterizations of prepared AR1-6 liposomes*

3.3.1. *Microscopic appearance*

The transmission electron photomicrographs (TEM) of AR1-6 liposomes having LEC or PC90G as structural lipid are illustrated in Figs. 2 and 3, respectively. The existence of the phospholipid bilayers could be evidenced by the concentric lamellar structure of vesicles as shown in the TEM, which appeared to be the oligolamellar type for liposomes prepared by REV and PD, and multilamellar vesicles for those prepared by CF and MFD. The differences in lamellar structure and vesicle type could be attributed to different mechanisms of vesicle forming by these methods of preparation.

Table 1 – R_f values and color zones of TLC fingerprints of DG and AR1-6 run by using system I under visible light.

Compounds	R_f	Color under visible light
DG	0.39	Dark yellowish green
AR1-6	0.10	Grey
	0.22	Light blue
	0.39	Light yellowish green
	0.47	Light blue
	0.59	Light purple
	0.73	Light purple
	0.79	Blue
	0.86	Purple

3.3.2. *Particle size*

It can be seen in Table 2 that the average particle sizes of AR1-6 liposomes were within the range of approximately 0.2–13.9 μm, depending on their lipid types and methods of preparation. No attempt was made to reduce the size or restrict the size distribution of liposomes. The broad distribution width, indicated by 'Span', of some formulations such as those with LEC and prepared by CF can be considered as the strong aggregation of some hydrated vesicles. The smallest one was the liposomal formulation having PC90G as structural lipid and prepared by PD, whereas the largest one was those with PC90G and prepared by MFD.

AR1-6 liposomes having LEC as structural lipid and prepared by all methods except PD exhibited smaller particle size than those with PC90G. Such results could be attributed to the difference in purity and degree of saturation including the carbon number of the acyl hydrocarbon chains in the phospholipids used, in which PC90G is of a higher purity (94–102%) than LEC (63%) [22]. Besides, incorporation of phospholipid with higher degree of saturation into liposomes would yield larger particles because of the reduction in fluidity or microviscosity of the bilayer. This would enhance the rigidity of the bilayer membrane above the phase transition temperature, resulting in an increased elastic modulus and inhibited curving of the bilayer. Likewise, the unsaturated phospholipids exhibited the conical shape while the saturated ones exhibited the cylindrical shape. Association of such conical shape molecules was weaker than that of cylindrical shape molecules due to a longer intermolecular distance, which resulted in a higher fluidity of phospholipids containing unsaturated phospholipids. As expected, vesicles containing unsaturated phospholipids would result in smaller particle size than that of saturated ones [22].

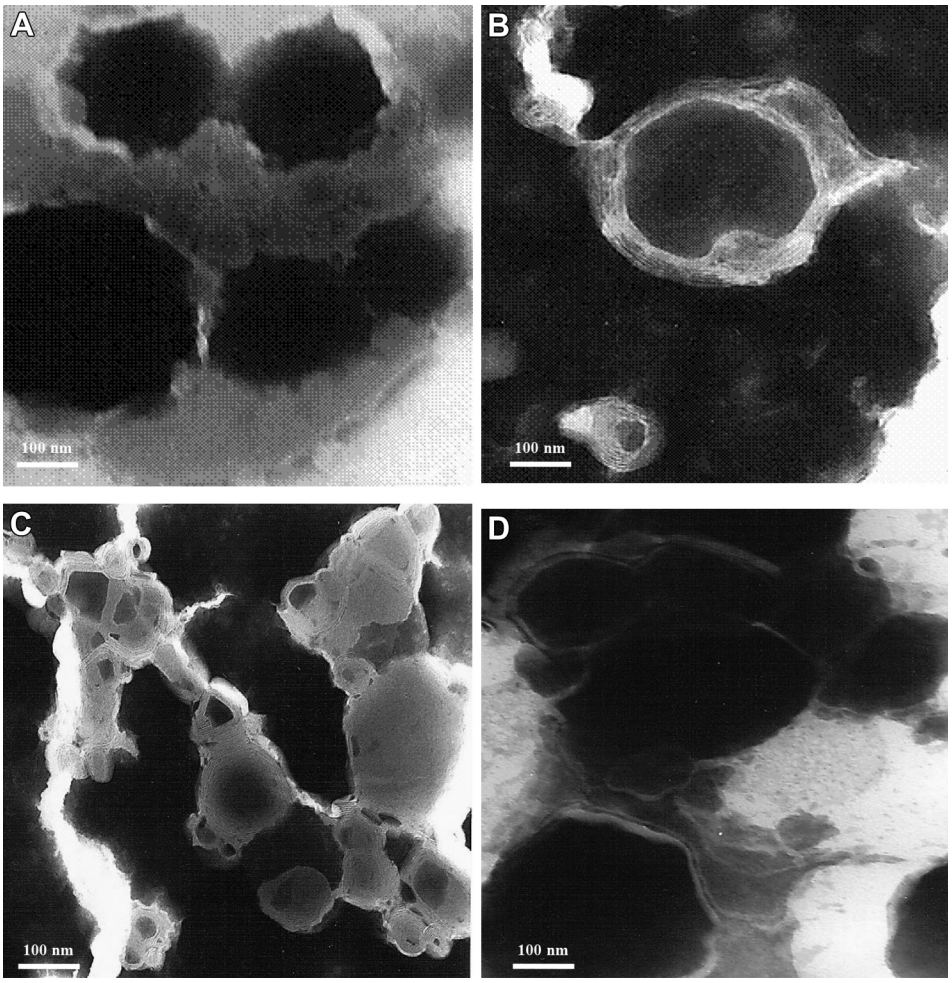

Fig. 2 – TEM photomicrographs of prepared AR1-6 liposomes comprising AR1-6 to lipid weight ratio of 1:10 and LEC to CHOL molar ratio of 7:3 prepared by CF (A), REV (B), PD (C), and MFD (D) methods (magnification 75,000×).

However, the results were completely opposite for vesicles prepared by PD, in which propylene glycol added would have affected the hydrophilic-lipophilic balance (HLB) of the system, and hence, the change in liposome size accordingly [23]. It has been described that emulsifiers of high HLB values always formed microsystems with smaller isotropic regions than ones of lower HLB values [24]. As a result, the mean size of PD liposomes composed of LEC with higher HLB value appeared to be larger than those of PC90G.

In order to investigate the effect of method of preparation on particle size, the obtained liposomes were not subject to further size reduction. It is clearly demonstrated that the average particle sizes of vesicles with LEC were found to be significantly different in rank order as follows: PD > MFD > CF > REV ($P < 0.05$, ANOVA), whereas those with PC90G were significantly different in rank order as follows: MFD > REV > CF > PD ($P < 0.05$, ANOVA). Such behaviors may be attributed to the differences in vesicle type and lamellar structure of liposomes prepared by these four methods due to different mechanisms of vesicle forming. Generally, liposomes prepared by CF and MFD exhibited multilamellar type whereas those prepared by REV and PD exhibited oligolamellar in nature. In fact, multilamellar vesicles mostly possessed quite larger particle size than oligolamellar ones did.

3.3.3. *Particle surface charge*

It was found that zeta potentials of AR1-6 liposomes prepared by these four methods were negative charge in nature within the range of −1.5 to −39.3 mV as shown in Table 2, which would ensure repulsive force to prevent them from aggregation. Statistical testing revealed that the zeta potentials of AR1-6 liposomes having LEC were significantly higher negative than those having PC90G for each method of preparation ($P < 0.05$, unpaired *t*-test). These results suggested that LEC in medium at neutral pH may have a negative charge depending on its degree of impurity, whilst PC90G will have a zero charge. Therefore, the slightly negative zeta potential of liposomes was due to the presence of small amount of other negatively charged phospholipids (phosphatidylserine, phosphatidylinositol) [25].

Speaking in terms of the effect of preparation method, the rank order of negative zeta potentials of liposomes having PC90G were as follows: REV > CF > PD > MFD, with statistically significant difference ($P < 0.05$, ANOVA). It is also interesting to note that liposomes with PC90G and prepared by MFD were found to be least negative compared to other methods,

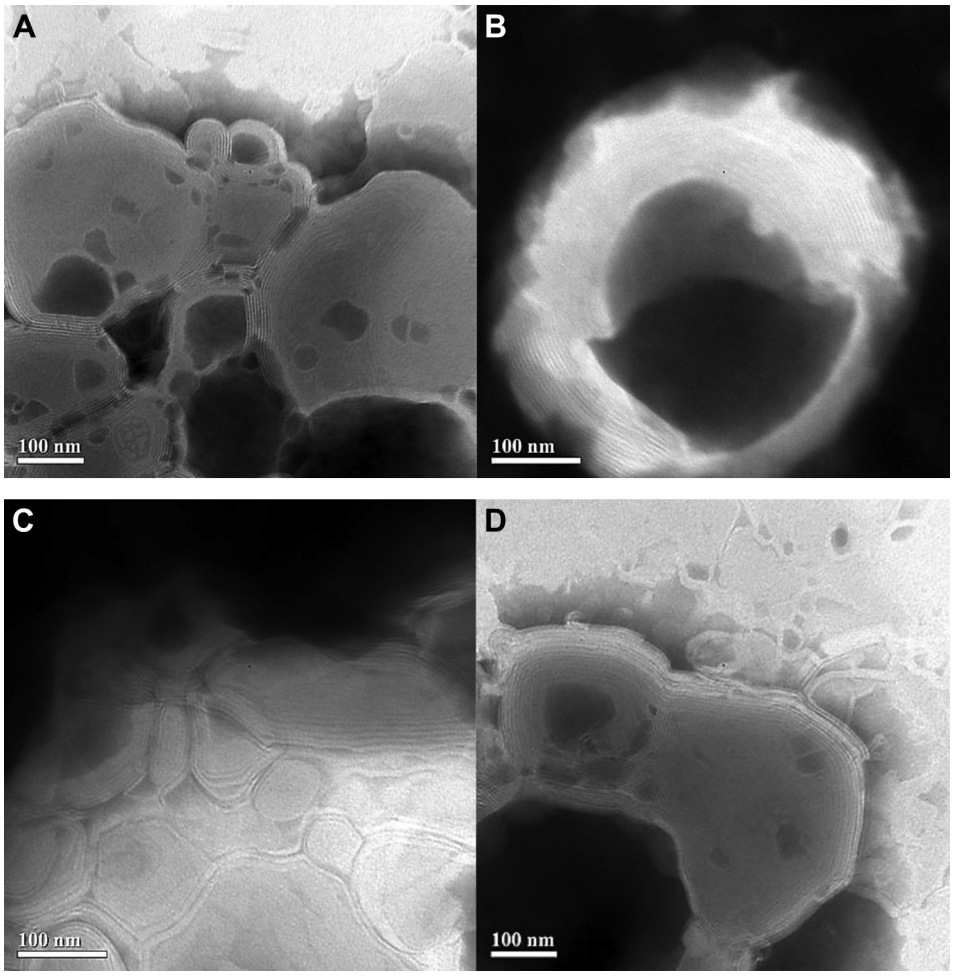

Fig. 3 – TEM photomicrographs of prepared AR1-6 liposomes comprising AR1-6 to lipid weight ratio of 1:10 and PC90G to CHOL molar ratio of 7:3 prepared by CF (A), REV (B), PD (C), and MFD (D) methods (magnification 75,000×).

reflecting the differences in drug localization in liposomes prepared by these methods aforementioned.

3.3.4. *Entrapment efficiency*

It was found that the minimum value of entrapment efficiency was 42.19% for liposomes having PC90G as structural lipid and prepared by REV, while the maximum one was as high as 69.08% for those with LEC and prepared by PD (Table 2). The presence of LEC as structural lipid resulted in a significantly higher drug entrapment efficiency than PC90G ($P < 0.05$, unpaired *t*-test). Since the AR1-6 molecule is positively charged at physiological pH, it could be expected to be

Table 2 – Particle size, zeta potential, entrapment efficiency, total saponin content in terms of diosgenin equivalent (DGE), and percentage of tyrosinase inhibition of AR1-6 encapsulated in liposomes comprising AR1-6 to lipid weight ratio of 1:10 and PC to CHOL molar ratio of 7:3 with various lipid types and methods of preparation.

Preparation method	Lipid composition	Particle size (μm) Mean (Span)[a]	Zeta potential (mV) Mean (SD)[a]	% Entrapment efficiency Mean (SD)[a]	DGE (μg/mg extract) Mean (SD)[a]	% Tyrosinase inhibition Mean (SD)[a]
CF	LEC:CHOL	3.24 (53.48)	−38.1 (0.3)	56.59 (0.72)	243.62 (1.51)	17.55 (1.14)
	PC90G:CHOL	6.32 (1.31)	−5.2 (0.5)	47.81 (0.69)	225.02 (1.61)	14.52 (0.57)
REV	LEC:CHOL	0.37 (3.92)	−36.0 (0.9)	48.19 (0.49)	223.61 (1.03)	14.98 (0.94)
	PC90G:CHOL	13.34 (2.25)	−6.1 (0.2)	42.19 (0.61)	210.51 (1.52)	12.68 (0.45)
PD	LEC:CHOL	8.67 (4.46)	−39.3 (0.4)	69.08 (0.64)	273.92 (1.40)	25.00 (0.93)
	PC90G:CHOL	0.26 (12.61)	−4.0 (0.1)	63.19 (0.51)	257.97 (1.23)	23.64 (1.20)
MFD	LEC:CHOL	7.20 (7.88)	−37.7 (0.6)	49.40 (1.05)	228.74 (2.44)	13.27 (1.13)
	PC90G:CHOL	13.83 (4.94)	−1.5 (0.1)	44.58 (0.14)	216.13 (0.35)	12.06 (0.80)

[a] Average from 3 determinations.

entrapped at higher amount in liposomes with more negatively charged surface by electrostatic interactions [26].

In addition, there was at least one pair having statistically significant difference among all values of entrapment efficiency for each lipid type LEC or PC90G ($P < 0.05$, ANOVA). It was further shown that almost all pairs of entrapment efficiency with various methods of preparation at the same lipid type were significantly different statistically ($P < 0.05$, LSD). The rank order of entrapment efficiency for vesicles with LEC was found to be the same as that with PC90G, i.e. PD > CF > MFD > REV. These results indicated that the heating process used in PD brought about significantly higher entrapment efficiency than the solvent technique such as CF and REV ($P < 0.05$, ANOVA). Since AR1-6 used in this study was practically soluble in aqueous medium, the liposomes prepared by PD should be more capable of encapsulating a higher mass of AR1-6. Besides, the PD did not involve the use of organic solvents [27].

3.3.5. *Total saponin content*

Total saponin content in terms of DGE's of AR1-6 in liposomes as shown in Table 2 were in the rank order of PD > CF > MFD > REV, with statistically significant difference ($P < 0.05$, ANOVA) for each lipid type. DGE of liposomes with LEC was significantly higher than those with PC90G for all pairs of samples with the same method of preparation ($P < 0.05$, unpaired *t*-test). It was apparently shown that AR1-6 liposomes having LEC as structural lipid and prepared by PD possessed the highest DGE value.

3.3.6. *Tyrosinase inhibitory activity*

The percentage tyrosinase inhibition–concentration profiles of kojic acid and AR1-6 are depicted in Figs. 4 and 5, respectively. It is interesting that AR1-6 exhibited substantial inhibitory effect on the activity of enzyme tyrosinase. The enzyme activity decreased dramatically with the increasing concentration of AR1-6. These results revealed that the inhibition of tyrosinase on the catalysis of the oxidation of L-DOPA displayed a dose-dependent manner. The IC_{50} values of kojic acid and AR1-6 were 15.22 ± 0.31 μg/ml and 6.76 ± 0.68 mg/ml, respectively, indicating that the value of AR1-6 was approximately 410–470

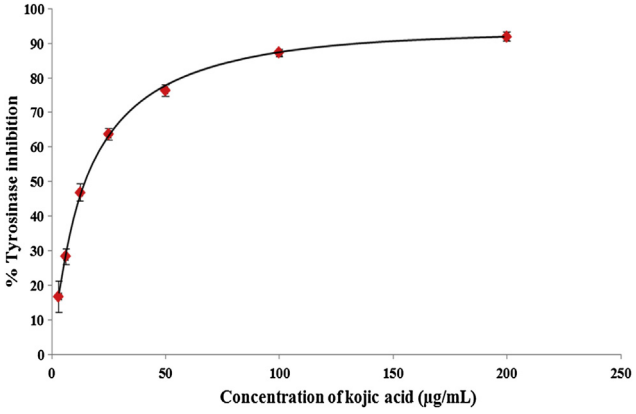

Fig. 4 – Percentage tyrosinase inhibition–concentration profile of kojic acid. Each data representing mean ± SD of 3 determinations.

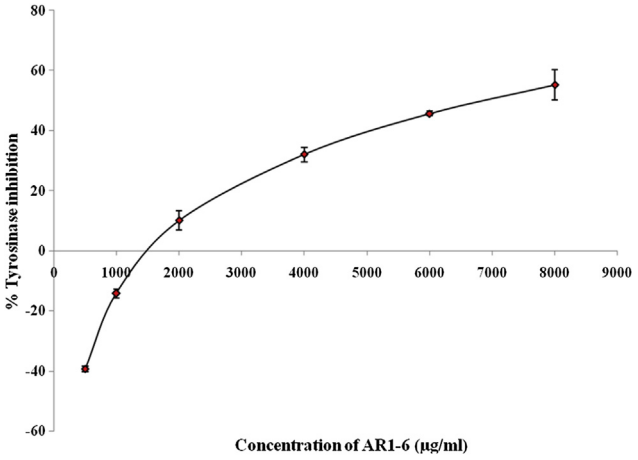

Fig. 5 – Percentage tyrosinase inhibition–concentration profile of AR1-6. Each data representing mean ± SD of 3 determinations.

folds lower than that of kojic acid, a potent tyrosinase inhibitor used as reference. The AR1-6 liposomes with LEC as structural lipid and prepared by PD possessed the highest tyrosinase inhibitory activity of 25.00% whilst those with PC90G and prepared by MFD exhibited the lowest activity of 12.06% (Table 2). In general, AR1-6 liposomes with LEC possessed higher inhibitory activity than those with PC90G, and the inhibitory activity could be ranked in the order based on the preparation method as PD > CF > REV > MFD. Such behavior could be explained by the same rank order relationship of entrapment efficiency and DGE of AR1-6 liposomes as mentioned above.

Generally, tyrosinase inhibitors either render the copper interaction within the active site to be inactive by chelation, obviating the substrate–enzyme interaction, or inhibit oxidation via an electrochemical process [28]. The inhibition exerted by kojic acid is well established to come from its ability to chelate the copper atoms in the active sites of the enzyme [29]. It has been reported that the major active constituents of *A. racemosus* are steroidal saponins (Shatavarins I–IV) present in the roots [4]. Besides, other active compounds such as quercetin, rutin, kaempferol, racemofuran, and isoflavone were also found and identified on the basis of chemical and spectroscopic evidence. It has previously been observed that a number of flavonoids were found to manifest the tyrosinase inhibitory activity with some flavonols acting as copper chelators [29]. Besides, quercetin and kaempferol were found to inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase. Although the inhibition mechanism of the, herein, crude ethanolic extract of *A. racemosus* roots was still unclear, it is possible that the active groups of this compound is thought to be hydroxyl group as well as the substituted furan ring (2,3-dihydro-2,3,3-trimethylfuran moiety) that help increase its inhibition capacity on mushroom tyrosinase. In addition, extensive studies of the chemical structures of kojic acid, kaempferol, and quercetin revealed the structure similarity especially the 3-hydroxy-4-keto moiety, which is the important substructure to react with the copper ions of tyrosinase.

4. Conclusion

AR1-6 liposomes with LEC or PC90G as structural lipid could be prepared by CF, REV, PD, and MFD methods. The obtained liposomes possessed the size range of 0.26–13.83 μm and slightly negative charge of −1.5 to −39.3 mV, whereas the entrapment efficiency and *in vitro* tyrosinase inhibitory activity were 42.19%–69.08% and 12.06%–25.00%, respectively. Types of lipid and preparation methods significantly influenced the physicochemical properties of liposomes such as vesicle type, size, surface charge, drug entrapment, and biological activity.

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