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# FORMULATION AND EVALUATION OF NIOSOMES ENCAPSULATED WITH KT2 AND RT2: ANTIMICROBIAL AND ANTICANCER PEPTIDES DERIVED FROM CROCODILE LEUKOCYTE EXTRACT

Tinnakorn Theansungnoen *, Jureerut Daduang, Aroonsri Priprem, Patcharee Boonsiri, Sakda Daduang and Sompong Klaynongsruang

Green Cosmetic Technology Research Group, School of Cosmetic Science, Mae Fah Luang University, Chiang Rai 57100, Thailand.

**Keywords:**

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**Correspondence to Author:**
**Dr. Tinnakorn Theansungnoen**

Lecturer,
Green Cosmetic Technology Research Group, School of Cosmetic Science, Mae Fah Luang University, Chiang Rai 57100, Thailand.

**E-mail:** tinnakornthean@gmail.com

**ABSTRACT:** Niosome is one of the synthetic nanoparticles which often is used for drug delivery and cosmetic industry. The aim of this study was to prepare niosomes encapsulated with KT2 and RT2. KT2 and RT2 are antimicrobial and anticancer peptides derived from crocodile leukocyte extract. Niosomes encapsulated with KT2 and RT2 were prepared by thin-film hydration technique, and peptide encapsulation efficiency was evaluated. Niosome particle size, size distribution, and surface morphology were also studied. The results show that KT2 and RT2 were successfully loaded in the formulations with encapsulation efficiencies of 70.9 ± 0.9% and 59.6 ± 3.4%, respectively. The mean particle size and zeta potential of KT2 and RT2 encapsulated niosomes were 9.3 ± 1.13 µm and - 57.39 ± 2.53 mV and 9.1 ± 0.03 µm and - 50.91 ± 2.28 mV, respectively. Niosomes had spherical shapes with rough surfaces. Niosomes coated with hydroxypropyl methylcellulose phthalate (HPMCP) were evaluated for pH stability. At pH 1.2 and 4.5, the peptide-encapsulated niosomes were stable. In conclusion, these peptide-encapsulated niosomes have high potential for therapeutic, cosmetic and beauty product applications.

**INTRODUCTION:** Nanoparticles have been developed as an effective carrier in transporting drugs through the human body since the small particles can show enhanced properties including high reactivity, surface area, strength, sensitivity, stability, and so on [1]. Niosome is a synthetic vesicle of nanoparticles, which is formed on the mixture of non-ionic surfactant(s) and cholesterol with subsequent hydration in aqueous media, and also serves as a transporter in drug delivery system. Hydrophilic drugs can be encapsulated in niosome while hydrophobic agents are embedded within the bilayer of the vesicles. In medical application, niosome has been used as a delivery system of anticancer compounds (curcumin [2] and paclitaxel [3]) and antimicrobial compounds (moxifloxacin [4], bismuth-ethanedithiol, and tobramycin [5]). Moreover, niosome can encapsulated proteins or peptides entrapped to protect from degradation by proteolytic enzymes [6, 7].

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KT2 and RT2 are antimicrobial peptides, rich in arginine and lysine residues, from crocodile (*Crocodylus siamensis*) leukocyte. KT2 and RT2 provide strong efficacy to kill both Gram-positive bacteria (*Staphylococcus epidermidis* ATCC 12228) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella typhi* DMST 22842,

and *Vibrio cholerae* non-O1, non-O139) [8]. Moreover, KT2 and RT2 can prevent biofilm formation of Gram-negative *E. coli* O157: H7 by inhibiting cell proliferation and also kill mature cells of the bacteria after biofilm formation [9]. KT2 and RT2 may kill bacteria by disruption on bacterial membrane and/or translocation into cytoplasm of the cell. They inhibited Hela and CaSki cancer cell proliferation by stimulating apoptosis [10] but did not affect RAW 264.7 mouse macrophage cells, monkey Vero kidney epithelial cells, and human red blood cells [8]. According to the previous studies, KT2 and RT2 had high potential as alternative agents for medical and cosmetic applications. Therefore, the aim of this study was to produce niosomes encapsulated with KT2 and RT2. The peptide-encapsulated niosomes were characterized in terms of the encapsulation efficiency, particle size and potential, surface morphology and pH stability.

## MATERIALS AND METHODS:

**Peptides and Materials:** Peptide sequences (KT2 and RT2) were synthesized by solid-phase methodology with Fmoc-protected amino acids (GL Biochem, Shanghai, China). The purity of the peptides products was verified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) as greater than 95%. Sorbitan monostearate (Span 60) was purchased from Sigma-Aldrich (United States). Cholesterol was obtained from Fluka Chemie (GmbH, Japan). Chloroform, methanol, and ethanol were purchased from RCI Labscan (Thailand). All materials used in the study were of analytical grade.

**Preparation of Peptide-Encapsulated Niosomes:** Niosomes encapsulated with KT2 and RT2 peptides were prepared by a thin-film hydration technique with slightly modification from the previous study [11]. The surfactant Span 60 (73 mg) and cholesterol (65 mg) were mixed into a 100 ml-round bottom flask and dissolved in 3 ml of solvent mixture of ethanol and chloroform (2:1 v/v). The organic solvent was then evaporated and the resulting thin film was performed under vacuum pressure at 70 °C for 1 h, using a rotary evaporator (EYEL4, Eyela Tokyo Rikakikai, Japan) followed by air dry for 30 min. The resultant film was hydrated with 1 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.4 containing either KT2 or RT2 at 50 °C for 15 min. The niosomes were coated with hydroxypropyl methylcellulose phthalate (HPMCP), HP55. The niosome suspension was stored at 4 °C for further experiments.

**Encapsulation Efficiency Measurement:** The encapsulation efficiency (EE) of peptide-encapsulated niosomes was investigated, and the extraction condition of niosomes was modified from the previous study [12]. Briefly, 1 ml of niosome suspension was centrifuged at 15,000 rpm for 2 h at 4 °C. Niosome pellets were collected, and 10 µl was added to 0.2 ml of methanol (60% v/v), followed by mixing vigorously. After centrifugation at 15,000 rpm for 10 min, 0.1 ml of supernatant was used to determine the peptide content by using an Eppendorf BioSpectrometer (Eppendorf AG, Germany) at a wavelength of 220 nm. The percentage of encapsulation efficiency (EE%) of the peptide-encapsulated niosomes was calculated by using the following equation:

$$EE\% = (A/B) \times 100$$

Where A is the amount of peptide contents in niosomes and B is the amount of feeding peptides.

**Determination of Zeta Potential of Niosomes:** Zeta potential of peptide-encapsulated niosomes was measured, in six repeated times, by a dynamic light scattering (DLS) zeta potential analyzer (Zetasizer nano series, Malvern Instruments, United Kingdom) by controlling the count rate around 3,000 kcps of the particles. Briefly, 10 - 20 µl of niosome suspension was mixed with 3 ml of distilled water. About 1 ml of the diluted niosome solution was then filled into the cuvette in DLS zeta potential analyzer.

**Determination of Particle Size and Size Distribution of Niosomes:** Particle size and size distribution of peptides-encapsulated niosomes were measured, in six repeated times, by a laser beam based mastersizer (Mastersizer 2000, Malvern Instruments, United Kingdom). Niosome suspension (0.1 - 0.2 ml) was added into 75 ml of distilled water followed by mixing in a dispersion unit controller at 2000 rpm, 25 °C. The stirring water was circulated through the lens, and the particle size of niosomes was detected by the DLS of the laser beam at 633 nm.

**Characterization of Niosome Surface Morphology:** The surface morphology of the niosomes were characterized by using a scanning electron microscope (SEM). Niosome suspension (1 ml) was centrifuged at 12,000 rpm for 10 min. Niosomal pellets were washed twice with distilled water and re-centrifuged. The pellets were re-suspended with distilled water (0.4 ml). The sample (2 - 5 µl) was dropped on a covered glass slide and then air-dried. After coating with gold on the sample, the particle features were observed and the images were captured by a Quanta 450 scanning electron microscope (FEI, Eindhoven, The Netherlands).

**Determination of pH Stability of Niosomes:** The pH stability of peptide-encapsulated niosomes was evaluated. The buffers at the various pH were 0.2 M KCl-HCl solution (pH 1.2), 0.1 M phosphate buffer (pH 4.5), and 0.1 M phosphate buffer (pH 6.8). Niosomes were mixed individually in each buffer at the ratio of 1:12 (v/v), at room temperature. At the time interval of 5, 10, 15, 30, 45, 60, 120, and 180 min, 0.6 ml of sample was taken out and then centrifuged at 10,000 rpm for 1 min. The amount of each peptide sample was measured at 220 nm by using an Eppendorf BioSpectrometer (Eppendorf AG, Germany).

**Statistical Analysis:** All data are presented as mean ± SD. Comparisons among different groups were performed by analysis of variance using the ANOVA test, and significant difference calculations were carried out via the Student's t-test. The statistical significance of all values was considered at *P*-values less than 0.05 (*P< 0.05).

## RESULTS AND DISCUSSION:

**Formulation and Encapsulation Yield of KT2- or RT2- Encapsulated Niosomes:** KT2 and RT2 are antimicrobial and anticancer peptides derived from crocodile (*C. siamensis*) leukocyte extract [8-10]. Niosomal nanotechnology was used to stabilize the peptide. In this study, KT2 and RT2 were successfully encapsulated by using the formulation as shown in **Table 1**.

**TABLE 1: COMPOSITION OF THE NIOSOME FORMULATIONS**

| Formula code | Organic phase | | | | Aqueous phase | | |
|---|---|---|---|---|---|---|---|
| | Span 60 (mg) | Cholesterol (mg) | Ethanol (ml) | Chloroform (ml) | Peptide (µM) | | PBS (ml) |
| | | | | | KT2 | RT2 | |
| CON | 73 | 65 | 2 | 1 | - | - | 1 |
| KTN | 73 | 65 | 2 | 1 | 0.5 | - | 1 |
| RTN | 73 | 65 | 2 | 1 | - | 0.5 | 1 |

CON: niosome without peptide, KTN: KT2-encapsulated niosome, RTN: RT2-encapsulated niosome, PBS: phosphate buffer saline

The niosomal vesicles were prepared with cholesterol and Span 60 at the molar ratio of 1:1 *via* thin film hydration technique. According to some previous studies, the formulation of niosome vesicles was successful by using the combination of cholesterol and Span 60 [6, 13]. Span 60 is non-ionic surfactant, whereas cholesterol acts as an additive agent which can promote self-assembly into niosomes [14]. In addition, cholesterol can be used to provide rigidity and proper shape and good physical stability of niosome vesicles [15, 16]. The percentage of encapsulation efficiency (%EE) of niosomes was evaluated **Table 2**.

**TABLE 2: PARTICLE SIZE, ZETA POTENTIAL, AND % ENCAPSULATION EFFICIENCY (%EE) OF NIOSOMES COATED WITH OR WITHOUT HYPROMELLOSE PHTHALATE (HPMCP)**

| Formula code | Particle Size (µm) (mean ± SD) | Zeta Potential (mV) (mean ± SD) | %EE |
|---|---|---|---|
| CON | 6.3 ± 0.70 | -33.35 ± 2.13 | ND |
| KTN | 9.3 ± 0.13 | -57.39 ± 2.53 | 70.4 ± 0.90 |
| RTN | 9.1 ± 0.03 | -50.91 ± 2.28 | 59.6 ± 3.40 |
| CON_HP | 21.0 ± 1.08 | -30.28 ± 4.41 | NA |
| KTN_HP | 12.3 ± 1.10 | -69.39 ± 2.57 | NA |
| RTN_HP | 16.7 ± 0.88 | -72.18 ± 1.54 | NA |

CON: niosome without peptide, KTN: KT2-encapsulated niosome, RTN: RT2-encapsulated niosome, CON_HP: niosome coated with hypromellose phthalate, KTN_HP: KT2-encapsulated niosome coated with hypromellose phthalate, RTN_HP: RT2-encapsulated niosome coated with hypromellose phthalate, PBS: phosphate buffer saline, ND: not detected, NA: not assayed.

The encapsulation efficiency of KT2 and RT2 in the niosomes were 70.4% and 59.6%, respectively. The result reveals that the difference in encapsulation efficiency of peptides in niosomes

may cause their different solubility in PBS (pH 7.4). The different types of basic amino acid lysine (K) and arginine (R), which present in KT2 and RT2 respectively, may be a crucial key for the peptide solubility. Under physiological conditions, lysine and arginine, which have positive charge, play important roles in protein stability by forming electrostatic interaction and hydrogen bond in the proteins and also by interacting with water molecules [17, 18]. Arginine causes the protein structure to be more stable than lysine owing to its geometrical structure. The guanidinium group in arginine allows interaction in three directions through its three asymmetrical nitrogen atoms (Nε, Nη1, Nη2), whereas the basic functional group of lysine has only one direction of interaction [19, 20]. Therefore, arginine can form a number of interactions, such as salt-bridges and hydrogen bonds compared to lysine [21]. The salt interference from buffer may decrease the solubility of RT2 than KT2, leading to the undesired encapsulation efficiency of the peptides in niosomes.

**Particle Size and Size Distribution of KT2- or RT2- Encapsulated Niosomes:** Particle size and size distribution of peptide-encapsulated niosomes were evaluated in **Fig. 1**. Most niosomal particles of each formula showed a variety of particle sizes (0.1 - 100 µm). The results show that KTN (2 - 30 µm) **Fig. 1c** and RTN (2 - 30 µm) **Fig. 1e** had smaller size than CON **Fig. 1a** (0.12 - 40 µm), but the mean of particle sizes of KTN (9.3 µm) and RTN (9.1 µm) were larger than that of CON (6.3 µm) **Table 2**.

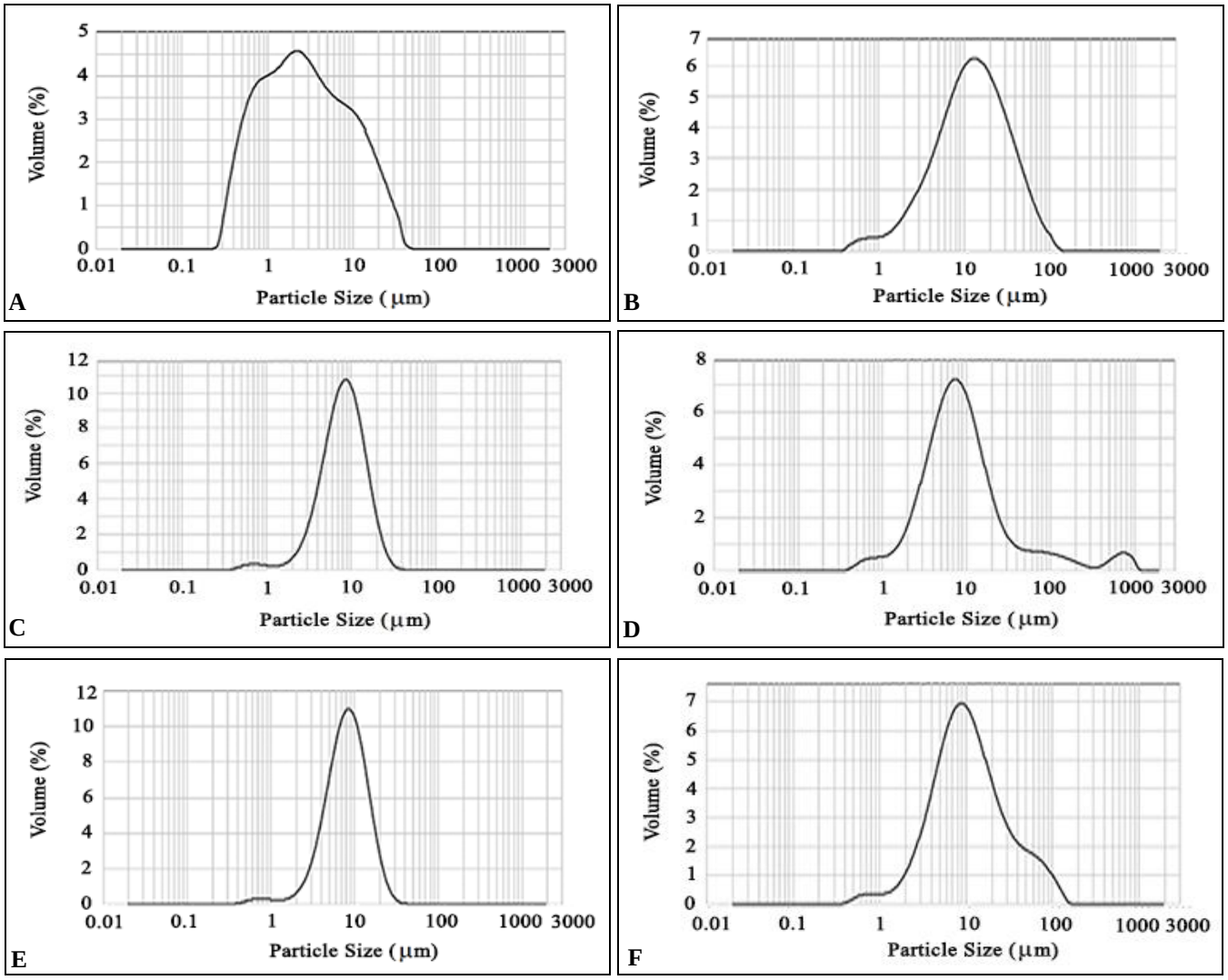


**FIG. 1: DISTRIBUTION OF PARTICLE SIZES OF EACH NIOSOME FORMULATION DETERMINED BY MASTERSIZER.** Ranges of various vesicle sizes were observed in (a) niosome without peptides (CON), (b) niosome coated with hypromellose phthalate (CON_HP), (c) KT2-encapsulated niosome (KTN), (d) KT2-encapsulated niosome coated with hypromellose phthalate (KTN_HP), (e) RT2-encapsulated niosome (RTN) and (f) RT2-encapsulated niosome coated with hypromellose phthalate (RTN_HP)

Formula CON_HP, KTN_HP, and RTN_HP showed the size distribution at 0.4 - 100 µm **Fig. 1b**, 0.4 - 120 µm **Fig. 1d** and 0.4 - 105 µm **Fig. 1f**, whereas their mean of particle sizes was 21.0 µm, 12.3 µm, and 16.7 µm, respectively **Table 2**. Particle sizes of CON_HP, KTN_HP, and RTN_HP were larger than uncoated niosomes, which indicated that niosomes were successfully coated with HPMCP.

Among various techniques of niosomal formation, thin-film hydration technique is widely used because it is simpler than the others. The examples of success studies by using the technique are the niosomes entrapped with temozolomide [22], etoricoxib [23], stavudine [24], cefixime [25], and piroxicam [26]. However, niosomes formed by the technique are multi-lamellar vesicles (MLVs) [27, 28].

To evaluate the stability of particles, formula KTN and RTN showed the zeta potential value at - 57.39 and - 50.91 mV respectively, whereas that of formula CON was - 33.35 mV **Table 2**. After coating with HPMCP, formulas KTN_HP and RTN_HP provided the zeta potential value at - 69.39 and - 72.18 mV. The results indicate that the peptide-encapsulated niosomes had sufficiently high electrostatic stabilization and their electrostatics could be increased after coating with HPMCP. It was reported that high negative zeta potential values of niosomes had the less possibility of particle aggregation, which suggested that the niosomal particles can be well-suspended in water [29]. This characteristic of niosomes is important for their long-term storage and applications, especially for cosmetic and beauty product formulation or therapeutic administration.

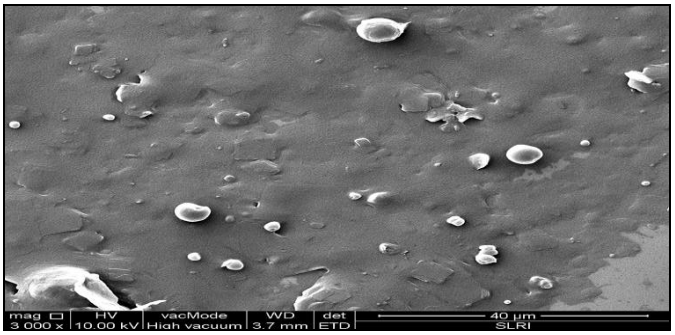

**FIG. 2: WHOLE VESICLES AND VARIOUS SIZE DISTRIBUTIONS OF NIOSOMES IMAGED BY SCANNING ELECTRON MICROSCOPY (SEM).** Single bar = 40 µm (magnification 3,000 ×)

**Morphological Evaluation of Niosomal Particles by Scanning Electron Microscopy:** In **Fig. 2**, the niosomal particles possessed the different sizes (<40 µm), which correlate to the results of particle size distribution in **Fig. 1**. Peptide-encapsulated niosomes of formulas KTN **Fig. 3c** and RTN **Fig. 3e** presented a spherical shape and very rough surface with white specks, whereas a spherical niosome without peptide of formula CON **Fig. 3a** had a smooth surface.

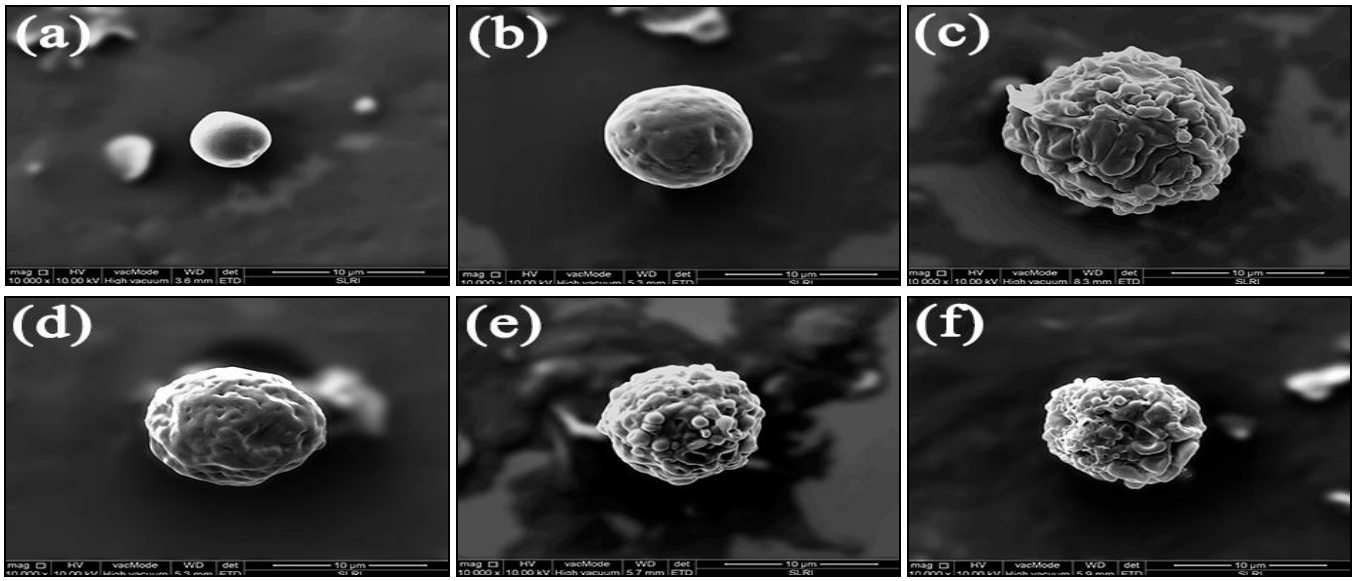


**FIG. 3: MORPHOLOGICAL SURFACE OF EACH SINGLE NIOSOMAL VESICLE OF NIOSOMES WITH OR WITHOUT HPMCP COATING CAPTURED BY SCANNING ELECTRON MICROSCOPY (SEM).** The images were (a) niosome without peptides (CON), (b) niosome coated with hypromellose phthalate (CON_HP), (c) KT2-encapsulated niosome (KTN), (d) KT2-encapsilated niosome coated with hypromellose phthalate (KTN_HP), (e) RT2-encapsulated niosome (RTN) and (f) RT2-encapsulated niosome coated with hypromellose phthalate (RTN_HP). Single bar = 10 µm (magnification 10,000 ×)

Among HPMCP-coated niosomes, formula's KTN_HP **Fig. 3d** and RTN_HP **Fig. 3f** were roughness spherical niosomes whereas formula CON_HP **Fig. 3b** was slightly roughness spherical niosome. Our results were supported by a previous study [30] that metformin hydrochloride-loaded niosomes, synthesized by Span 40 and cholesterol, showed spherical shape with a rough surface, especially MN2 which is metformin hydrochloride-loaded niosomes with addition of dicetyl phosphate (DCP) to the formulation for increasing the negative surface charge [30]. However, our results were different from the following previous studies. Piroxicam-loaded niosomes, created in the presence of cholesterol and Span 60, had the spherical shape with a smooth surface similar to a niosome only [31]. Niosomes with 2.5 and 0.5 mg/ml melatonin, formed by the mixture of Span 60 and cholesterol, and prepared by the thin film hydration showed the smooth surface, but melatonin niosomes coated with polycaprolactone (PCL) revealed the roughness and white specks on the surface [32].

***In-vitro* pH Stability of KT2- or RT2-Encapsulated Niosomes:** After 3-h incubation at various pH (1.2, 4.5 and 6.8), peptides were released from KTN **Fig. 4a**, KTN_HP **Fig. 4b**, RTN **Fig. 4c** and RTN_HP **Fig. 4d**. The peptide concentration releases of niosomes at pH 6.8 were significantly higher than that at pH 1.2 and 4.5. Among all formulas, the peptide release profiles of niosomes at pH 1.2 and pH 4.5 were not significantly different. Therefore, the results indicate that the peptide-encapsulated niosomes of all formulas had the potential to resist to pH 1.2 and 4.5. Moreover, the pH stability of both HPMCP-coated niosomes and uncoated niosomes were similar. In this study, cholesterol and Span 60 were selected as components of peptide-encapsulated niosomes for niosomal formulation.

Although, niosomes are reported to have more stable than liposomes, the pH effect on niosomes consisted of cholesterol and Span 60 further investigation is needed. Our results show that for all niosomal formulas examined, the peptide release was the most rapid at nearly neutral pH whereas their release was a few amount at acidic pHs and stable for 3 h. However, the results were different from the previous study of Kailash V *et al*., (2013) [33] which 5-6-carboxyfluroscein (CF) was entrapped into niosomes. CF efflux from niosomes of each formula was rapid at pH 2.0, decrease extremely at pH 4.0, and provided steady until pH 8.0.

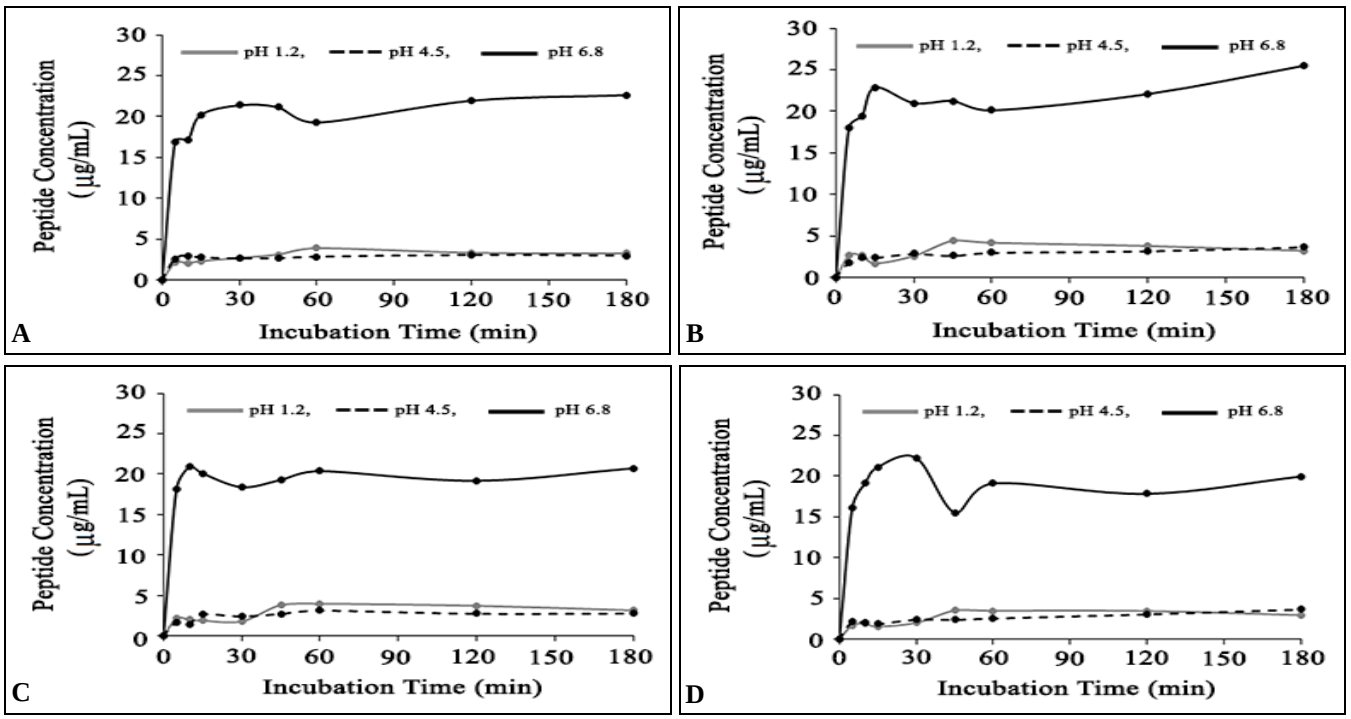


**FIG. 4: *IN-VITRO* STABILITY OF KT2- OR RT2-ENCAPSULATED NIOSOMES AGAINST VARIOUS GASTRIC PH.** The graphs were for (a) KTN, (b) KTN_HP, (c) RTN and (d) RTN_HP. Niosomes were incubated with gastric pH solutions including pH1.2, pH4.5 and pH6.8

In this study, the stability of peptide-encapsulated niosomes might result from Span 60 at membrane bilayer of the niosomal vesicles which pack tightly under acidic conditions resulting in an increased barrier to peptide releases.

**CONCLUSION:** The present study reports the success of encapsulation of crocodile blood-derived peptides KT2 and RT2 into niosomal vesicles by thin film hydration technique. The peptide-encapsulated niosomes mean particle sizes are 12 - 17 µm for HPMCP-coated niosomes and 9 µm for uncoated niosomes. By SEM, the morphology of niosomes was characterized as spherical vesicles with various rough and white specks on the surface. The niosomes have moderate ability to encapsulate peptides and show the great potential to resist acidic conditions.

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