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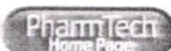


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Polymeric particulate system of carboxymethyl chitosan - diterpen lactone fraction of *Andrographis paniculata* nees : Characterization and *in vitro* release study

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Abstract: Objective: The objective of this research was to enhance the dissolution characteristic of diterpene lactone fraction of *Andrographis paniculata* Nees (DTLF) containing 75.9% andrographolide. The effect of carboxymethyl chitosan concentration on physical characteristics, entrapment efficiency and *in vitro* release of diterpene lactone fraction of *Andrographis paniculata* Nees (DTLF) particulate system were investigated.

Methods : Diterpene lactone fraction of *Andrographis paniculata* Nees (DTLF) particulate system with different amount of carboxymethyl chitosan (CMChi) were prepared by ionic gelation followed by spray drying methods with CaCl_2 as cross linker. The particles were evaluated in terms of physical state, drug entrapment efficiency and *in vitro* release rate.

Results : The result showed that DTLF is already entrapped in the system hence the crystallinity reduced. The entrapment efficiency increases with increasing the amount of carboxymethyl chitosan, between 84% up to 90%. The release rate of the particles in 0.5% SLS media were 1.5 times higher than DTL substance, but not significantly different as the polymer amount increased.

Conclusion : Entrapment of DTL in CMChi particulates system could reduce the crystallinity. Hence DTL – CMChi particles were able to enhance release rate of DTL up to 1.5 times.

Keywords: particulate system, carboxymethyl chitosan, diterpene lactone fraction, ionic gelation, spray drying.

Introduction

The number of substances that can be used as drug is increasing per year including substances that are obtained from the plant compounds. Nevertheless, more than 40% of these substances are poorly water-soluble hence limit their effectiveness due to their low bioavailability when given orally¹. *Andrographis paniculata* Nees is a plant that can be found throughout tropical and subtropical Asia, Southeast Asia and India¹⁸. One of the major phytoconstituents of this plant is andrographolide, which is a diterpenic lactone compound. This compound has been investigated to have broad pharmacological effects such as anti-inflammatory, antibacterial, antitumor, antidiabetic, antimalarial, and hepatoprotective^{2,3}. Similar with many other plant compounds, the major problem with this plant compound is its limited water solubility. The solubility of andrographolide in water is 3.29 $\mu\text{g/mL}$ and log P 2.632⁴. Pharmacokinetic study of andrographolide in rat and human showed that andrographolide underwent fast absorption and metabolism hence resulted in low bioavailability⁵.

One formulation strategy that can be applied to overcome poor water-solubility as well as poor bioavailability problem of drug substance is by formulating polymeric nanoparticles drug delivery. This system

consists of biocompatible and biodegradable polymers. The polymer forms a nanoparticle matrix with size range between 10-1000 nm and the drug is dissolved, entrapped, encapsulated or attached to the matrix⁶. In this system, the polymer also protects active ingredient from degradation in the biological system and increase its bioavailability by enhance its dissolution. Additionally, polymeric drug delivery can also be applied for targeted and sustained release drug delivery^{7,8,9}. With regard to the polymer applied, chitosan is one of the polymer that has been used widely to form polymeric nanoparticles. This polymer is biocompatible with living tissues and breaks down slowly as amino sugars, which is harmless and completely absorbed by the human body. In addition, this polymer is soluble in acidic water therefore the use of organic solvent can be omitted in the preparation process¹⁰. With regard to the chitosan, carboxymethyl chitosan (CMChi) is a water soluble derivate of chitosan and has similar biocompatibility as chitosan. CMChi is obtained from the carboxymethylation process, in which the –OH groups of chitosan are substituted by –CH₂COOH groups¹¹. This water soluble CMChi enables the preparation of polymer solution without the need to acidified the aqueous media. In general, there are several techniques that can be applied to produce CMChi polymeric nanoparticles, such as emulsion cross-linking, coacervation/precipitation, spray-drying, emulsion-droplet coalescence method, ionic gelation, reverse micellar method, and sieving method. Compared to the other available methods, ionic gelation method is a simple and versatile method since it can be applied without the use of organic solvent and high organic solvent¹². The principle of ionic gelation process is the reaction between the positive charge of the amino group of chitosan with negative charge of the polyanion cross-linker such as calcium chloride or tripolyphosphate. Both are in the aqueous form¹³. The spontaneous formation of nanoparticles takes place during the mixing and stirring of aqueous chitosan and aqueous cross-linker at room temperature. Furthermore, variation in the ratio of chitosan and stabilizer can modify the size and surface charge of the particles obtained¹⁴.

By far, oral drug delivery is the route of choice for most of drug administration with solid dosage form as the favorable dosage form. Hence, following the formation of polymeric nanoparticles, the aqueous polymeric nanoparticles obtained has to be solidified. Spray-drying is an efficient method to dry aqueous polymeric nanoparticles since this method is relatively fast compared to freeze-drying process. Additionally, spherical particles can be obtained by using this drying method¹⁵.

This study is aimed to optimize and characterize polymeric CMChi-DTL nanoparticles by using combination of ionic gelation-spray drying to enhance the dissolution rate of andrographolide in the DTLF. Influence of ratio between CMChi and CaCl₂ is also investigated in order to obtain optimum composition to produce a spheris polymeric nanoparticles.

Experimental

Materials

Andrographolide p.a (Merck KGaA, Germany), diterpene lactone fraction (DTLF) of *Andrographis paniculata* Nees containing 75.9% andrographolide (Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Indonesia), carboxymethyl chitosan (CMChi) with substitution degree 81.9% (China Easter Group Co.,Ltd., China), calcium chloride (CaCl₂) and ethanol (Merck KGaA, Germany) are in pharmaceutical grade.

Methods

Preparation of diterpene lactone fraction (DTLF) – carboxymethyl chitosan (CMChi) particulate system

Table 1. Composition of CMChi-DTLF particulates system

Formula	Amount (mg)		
	CMChi	CaCl ₂	DTLF
F1	200	80	40
F2	200	120	40
F3	200	100	40
F4	250	100	40
F5	300	100	40

Composition of the particulate system are detailed in Table 1. Preparation of the CMChi-DTLF nanoparticulate system was conducted by weighing 40 mg DTLF and dissolved it in ethanol. Following this, aqueous CMChi was mixed with ethanolic solution of DTLF and stirred at 500 rpm. Solution of CaCl_2 was then added into the mixture of CMChi-DTLF and stirred for 60 minutes. Following this crosslinking reaction, spray drying (SD-basic spray dryer SD B09060019, Lab Plant Ltd.,UK) was then conducted in order to solidify the mixture. The diameter of spray-dryer nozzle was 1.0 mm, inlet temperature was set at 100°C , pressure at 2 mBar and air flow rate was set at scale 3 of 10 of the spray dryer. The dried particles obtained from each mixture were then evaluated. Unloaded CMChi particles were also prepared to compare its physical characteristics with DTLF loaded particles.

Scanning Electron Microscopy

Scanning electron microscope (Inspect S50 Type FP 2017/12, FEI, USA) was used to determine the shape and size of the dried particles obtained either with or without DTLF. Particles were coated with gold palladium prior to analysis.

Fourier Transform Infrared (FT-IR)

Sample was made as a pellet by mixing 2 mg sample with 300 mg KBr powder then pressed with hydraulic pump to form a transparent pellet. Sample observation was conducted at wavelength $4000\text{--}450\text{ cm}^{-1}$ (Jasco FT-IR 5300, Easton MD, USA).

Differential Thermal Analysis

Approximately 5 mg of dried powder was placed in a crucible pan, sealed and observed for its thermogram. The thermogram was recorded at temperature 50 to 250°C with heating rate $10^\circ\text{C}/\text{min}$ using the Differential Thermal Analyzer (DTA FP-65 P-900 Thermal, Mettler Toledo, USA).

X-Ray Diffraction

Crystallinity of each sample was analyzed by using X-ray diffractometer (X'Pert analytical, The Netherlands). The light source employed was $\text{K}\alpha$ Cu Ni. The voltage and the current were set at 40 kV and 40 mA. Samples were analyzed at 2θ and angle between $5\text{--}40^\circ$.

Drug loading and entrapment efficiency

HPLC method was used to analyze the drug loading of DTL in CMCHI- CaCl_2 cross-linked nanoparticles. 5 mg sample was dissolved in 10 ml ethanol then filtered and analyzed with HPLC. The mobile phase consists of methanol: orthophosphoric acid pH 3 = 50:50. The flow rate was set at $0.75\text{ }\mu\text{l}/\text{min}$. The sample was measured at wavelength 228 nm. The assays were performed in triplicate. The drug loading and entrapment efficiency were calculated by using the following equations:

$$\text{Drug loading} = \frac{\text{drug amount}}{\text{particle weight}} \times 100\%$$

$$\text{Entrapment efficiency} = \frac{\text{actual drug amount}}{\text{theoretically drug amount}} \times 100\%$$

In vitro drug release

The medium used for *in vitro* drug release test was 0.1% w/v sodium lauryl sulfate (SLS). The test was conducted in 50 mL of this medium and the temperature was kept at $37 \pm 0.5^\circ\text{C}$ in water bath shaker. DTLF-CMChi particles equivalent with 2.0 mg FDTL was weighed accurately and sprinkled in the medium. 1.0 ml sample was taken at a predetermined time during 2 hours and analysed with HPLC. Replacement of the medium was done at each sampling time with the same volume of the withdrawn sample. Sample were analysed by HPLC methods as mentioned above.

Results and Discussion

Scanning Electron Microscopy

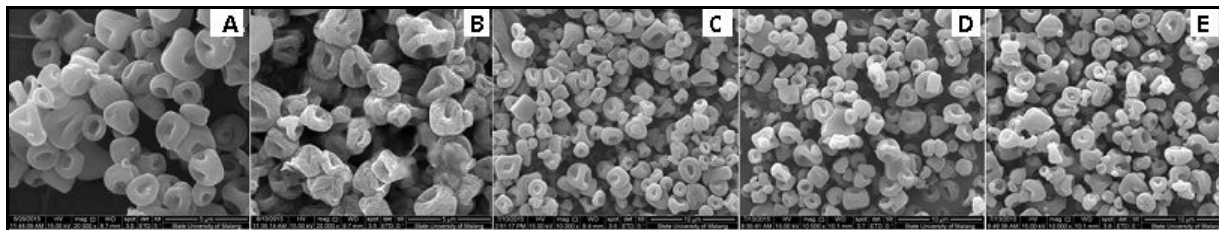


Figure 1. SEM of dried particles obtained from crosslinked: (A) CMChi:CaCl₂ = 200 : 80; (B) CMChi:CaCl₂ = 200 : 120; (C) CMChi:CaCl₂ = 200 : 100; (D) CMChi:CaCl₂ = 250:100; (E)CMChi:CaCl₂ = 300 : 100. Magnification of SEM (A) and (B) was 20.000x, SEM (C-E) was 10.000x.

Figure 1 showed the dried particulate system of cross-linked CMChi-CaCl₂ without DTLF. As observed, the particle shape is round and smooth with ring-like shape. Furthermore, when the DTLF was incorporated to form cross-linked polymeric particles, the surface of the particle became rough and the ring shaped was rarely observed. In addition, some non-round particles were also noticed. This is due to the DTLF that merged and packed inside the polymeric system therefore reduced the cross-link intensity.

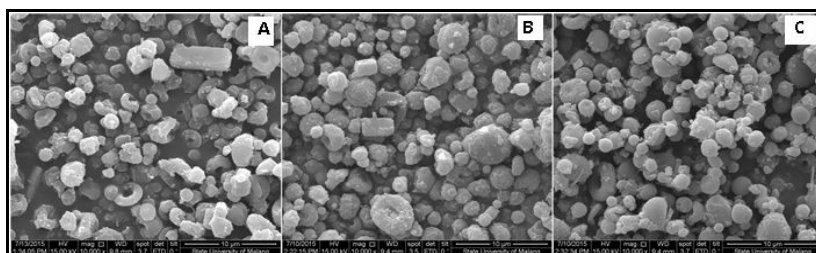


Figure 2. SEM of dried drug loaded particles with CMChi:DTLF:CaCl₂ratio (A) 200:100: 40, (B) 250:100:40, (C) 300:100:40. (Magnification 10.000x)

As seen in Figure 2, increasing concentration of CMChi in CMChi-DTLF nanoparticulate system resulted in more packed and spherical shape of the nanoparticles but with rough surface. However, the particle size obtained was varied from nanometer to micrometer scale since the drying process could not trap all small particles.

Fourier Transform Infrared (FT-IR)

Figure 3 shows shifting of the absorption bandwidth of -OH and -NH group of nanoparticulate system CMCHI-CaCl₂ when compared to the absorption bandwidth of CMCHI. Absorption bandwidth of -OH and -NH group of CMCHI only was observed at wavelength number 3467 cm⁻¹ whilst this absorption bandwidth was shifted to 3432 cm⁻¹ in the nanoparticulate system. Additionally, absorption bandwidth of -COO⁻ symmetric group and -COO⁻ of asymmetric group were shifted from 1450 cm⁻¹ to 1425 cm⁻¹ and from 1650 cm⁻¹ to 1654 cm⁻¹, respectively. This indicates that both of these groups, -COO⁻ (carboxyl), -NH⁺ (amino), and -OH (hydroxyl) took part in the crosslinking reaction of CMChi and CaCl₂. This also similar with the previous finding from Cai et al.¹⁶. Furthermore, as shown in Figure 3, different amount of polymer in the particulate system also revealed similar phenomenon. In the interaction between DTLF-CMChi, increasing the content of polymer resulted in the sharper vibration of -OH, -NH and -COO group. This is due to the increase concentration of CMChi whilst the amount of CaCl₂ remained the same. Therefore, more free COO⁻ group was available and increased the absorption intensity.

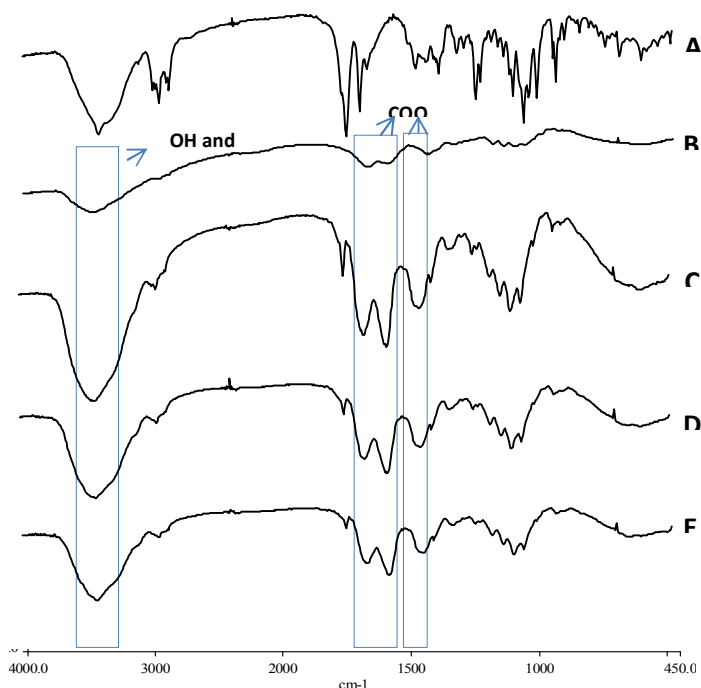


Figure 3. The infrared spectrum of: (A)DTLF; (B) CMChi; (C) CMChi : CaCl₂ : DTLF = 200:100:40; (D) CMChi : CaCl₂ : DTLF = 250 : 100 : 40, and (E) CMChi: CaCl₂ : DTLF = 300 : 100 : 40.

Differential Thermal Analysis

Figure 4 shows results for the thermal analysis of nanoparticulate system with and without the presence of DTL. In general, nanoparticulate systems obtained have lower melting point with less sharp peaks of CMChi compared to either CMChi or DTL substance. Additionally, in the nanoparticulate system containing DTL, endothermic peak of DTL was not observed. This indicates that the DTLF has been trapped in the nanoparticulate system. Interestingly, thermogram of unloaded particulate system with CMChi:CaCl₂ = 250 : 100 showed sharpest peak of CMChi compared to the two other combinations (200 and 300).

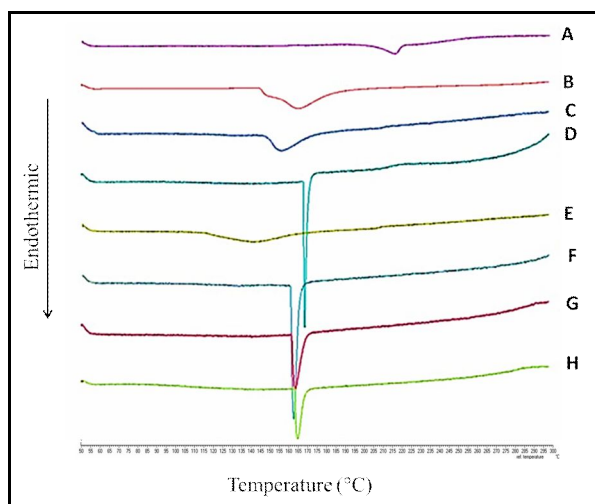


Figure 4. Thermogram of (A)DTLF, (B)CMChi, (C)CMChi:CaCl₂:DTLF=200:100:40; (D)CMChi:CaCl₂:DTLF= 250:100:40; (E)CMChi:CaCl₂:DTLF=300:100:40;(F)CMChi:CaCl₂= 200:100; (G) CMChi:CaCl₂ = 250:100; (H) CMChi:CaCl₂ = 300 : 100

Either with the presence of DTLF or without DTLF, both showed the same pattern. This indicates more ordered pattern conformation of the molecular system of CMChi:CaCl₂ at ratio 250:100 compared to the two

other compositions with CMChi 200 and 100, respectively. Increasing the CMChi to 300 and incorporating DTLF into this system caused shifting of the CMChi peak and also broadening of the peak.

X-Ray Diffractometry

As shown in Figure 5, main crystalline peaks of DTLF are shown at 2θ 9°, 12°, 14°, 15°, 17°. Peak of CMChi are shown at 2θ 19° but with weak intensity. Diffractogram of CaCl_2 showed one main peak at around 14°. It is interesting to notice that all nanoparticulate system of CMChi- CaCl_2 without DTLF (Figure 5.8 E, G, and I) showed one main new peak at 2θ 31°. Neither CMChi peaks nor CaCl_2 peak was observed in this particulate system. This indicates that the crosslinking between CMChi and CaCl_2 was already formed.

Furthermore, in the particulate system of DTLF-CMChi, crystalline peak of DTLF were not observed but the specific new peak of CaCl_2 at 2θ 31° was appeared in all formulation (F3 to F5). This proves that DTLF is already entrapped in the system hence the crystallinity reduced. Additionally, particle size of DTLF decreased and dispersed in the system. The reduction in the crystallinity and particle size give beneficial effect in increasing the dissolution of the DTLF that poorly soluble in water.

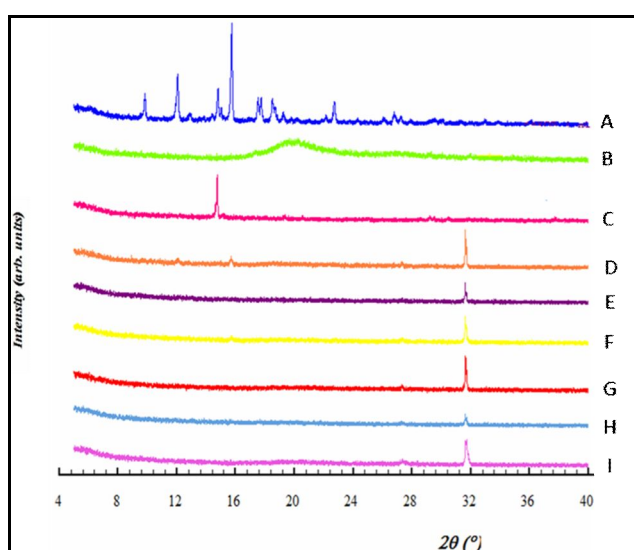


Figure 5. X-ray diffractogram of: (A) DTLF; (B) CMChi; (C) CaCl_2 , and nanoparticulate of CMChi- CaCl_2 -DTLF of formula: F3 (D), F4 (F), F5 (F). (E), (G), (I): Nanoparticulate system of CMChi- CaCl_2 without DTLF:

Drug Load and Entrapment Efficiency

HPLC analysis of DTLF content in nanoparticulate system showed that increasing the amount of CMChi (F3, F4 and F5) increased the amount of DTLF entrapped. Nevertheless, increasing concentration of CMChi higher than 250 mg did not increase the amount of DTLF entrapped in the system. This indicates that higher concentration of polymer increases the density of the system and formation of crosslinking but when the density is too high, the crosslinking system is too tight therefore hinders the drug entrapment in the system.

Table 2. Drug content, entrapment efficiency (EE) and slope of DTLF of particulate system (n=3)

Formula	DTLF content (% average \pm SD)	Yield (% average \pm SD)	Slope (mg/ml.min ^{1/2})
F3	10.00 \pm 0.10	84.77 \pm 0.85	10,8060 \pm 0,4508
F4	9.23 \pm 0.12	90.51 \pm 0.15	11,9353 \pm 0,1160
F5	8.19 \pm 0.07	90.96 \pm 0.73	11,4407 \pm 0,5581

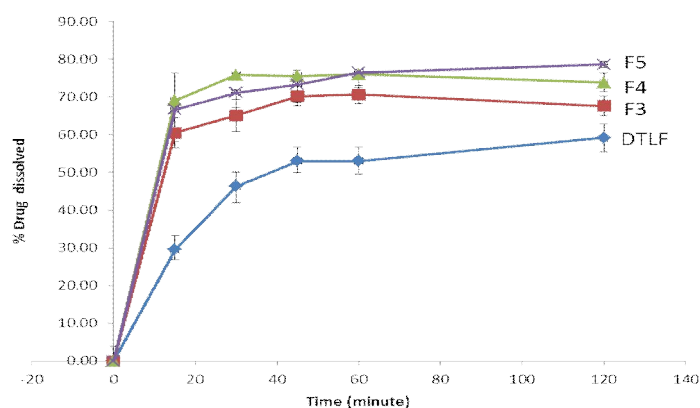
In vitro drug release

Figure 6. *In vitro* release of CMChi-DTLF particles and DTLF substance in 0,1 % SLS at $37 \pm 0,5^{\circ}\text{C}$.

In vitro release evaluation profile in Figure 4 indicated that % dissolved of DTLF from CMChi particulate system were greater than DTLF substance. Entrapment DTLF in CMChi particles affected the dissolution of DTLF. The slope value of DTLF was $8.0870 \pm 0.584 \text{ mg/ml.min}^{1/2}$ since the slope value of DTLF-CMChi particulate systems increased up to 1.5 times (Table 3). Spray drying can produce spherical and amorphous small particles that result from the rapid evaporation of solvent. The increase of surface area of particles and decrease of crystallinity can enhance the dissolution rate of poorly soluble drug¹⁷. This conformed with the results of DTA and X-ray evaluation in Figure 4 and Figure 5 that DTLF crystallinity in particulate systems decreased resulted in an increase of DTLF dissolution.

Conclusion

From this study it was concluded that drug-polymer ratio affected the entrapment efficiency but not the release rate. Carboxymethyl chitosan particulate system could enhance the release rate of DTLF of *Andrographis paniculata* Nees, containing 75,9% andrographolide which is poorly soluble.

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