ABSTRACT

Introduction

According to the WHO report 2014, 17.1% of all essential medicines are classified as BCS II (high permeability and low solubility) and 10.6% of them are classified as BCS IV (low permeability and low solubility). A drug can be absorbed by the body and provide the pharmacological effect for the body if the drug in a dissolved state.[1] Thus a low solubility drug results in low bioavailability.

Andrographolide, a diterpene lactone obtained from *Andrographis paniculata* has extensive pharmacological effects such as anti-inflammatory, anti-diarrhea, anti-HIV, anti-malarial, hepatoprotective, anticancer, antioxidant and antihyperglycemic. Andrographolide application is restricted due to its low water solubility, short half-life time (2 hours) and low permeability. Pharmacokinetic studies showed that andrographolide was quickly absorbed and metabolized in rats and humans.[2,3]

Several methods have been used to improve the low solubility of andrographolide. These methods are chemically modification, solid dispersion, liposomes, and nanoparticles. By improving the solubility, it is expected to increase the release rate and bioavailability.[2,4] Formation of nanoparticles andrographolide-eudragit® EPO increased bioavailability 2.2 times compared with pure andrographolide on oral administration.[5] Nanoparticles are a dispersion of solid particles with a diameter ranging between 10-1000 nm consisting of drug which dispersed, trapped or enveloped in a matrix of nanoparticles. Nanoparticles can be used as a drug carrier in drug therapy or vaccine adjuvant because of its small size can increase the absorption of the drug into the biological membrane by facilitated diffusion.[6,7,8]

Carboxymethyl chitosan is derivative of the water-soluble chitosan having amine and carboxyl groups in the molecule can be used as a carrier in drug delivery systems because these materials are biocompatible, biodegradable, non-toxic.[9] Carboxymethyl chitosan nanoparticles can be prepared by emulsification, gelation ionic, coacervation, spray drying, sonication, emulsion droplet-coalescence, reverse micellar and sieving. The mechanism of formation of carboxymethyl chitosan nanoparticles with an ionic gelation method is based on the electrostatic interaction between carboxyl groups of carboxymethyl chitosan and a positive charge of the crosslinker is $CaCl_2$. The addition of $CaCl_2$ is intended to form a bond between the divalent cations Ca^{2+} ions of $CaCl_2$ with -COO- of carboxymethyl chitosan.[9,10]

Malaria is an infectious disease caused by the parasite Plasmodium which is characterized by fever, anemia, and splenomegaly.[11] There are 198 million cases of malaria and 584 thousand deaths (78% children under 5 years of age) due to malaria worldwide by 2013. Antimalarial drug resistance such as chloroquine and sulfadoxine-pyrimethamine is a problem result in increased on morbidity and mortality caused by malaria.[12] Therefore, the development of antimalarial drug delivery systems still needs to be done.

In the present study, carboxymethyl chitosan nanoparticle of andrographolide was made by ionic gelation–spray drying to improve andrographolide physical properties and its antimalarial activity. The particles obtained were evaluated for its morphology, physical state, *in vitro* release and *in vivo* antimalarial activity on *Plasmodium berghei* infected mice.

Experimental

Material

Andrographolide (RD Health Ingredients Co., Ltd.); Carboxymethyl chitosan (degree of substitution 81.9%, 96.5% degree of deacetylation, viscosity 1% 22 mPa.s, China Eastar Group Co., Ltd.); Calcium chloride $CaCl_2.2H_2O$ pro analysis (Merck); methanol; 96% ethanol pro analysis; distilled water; *Plasmodium berghei* ANKA strain obtained from the Eijkman Institute for Molecular Biology, Jakarta and maintained SATREPS ITD (Institute of Tropical Disease); Alceivers medium; Giemsa dye in phosphate buffer; absolute methanol.

Preparation of andrographolide - carboxymethyl chitosan nanoparticles

The andrographolide – carboxymethyl chitosan nanoparticles were prepared by ionic gelation method using $CaCl_2$ as crosslinker then spray dried. 250 mg carboxymethyl chitosan was dissolved in 100 ml distilled water. Carboxymethyl chitosan solution was then poured into andrographolide solution (40 mg in 5 ml methanol) and stirred briefly at 500 rpm. Andrographolide-carboxymethyl chitosan solution was added into $CaCl_2$ solution (100 mg in 40 ml ethanol-water =1:9) and the mixture solution was stirred constantly for 4 hours at 500 rpm. Non-crosslinked particles were also prepared by simply mixed the polymer solution and andrographolide solution. Dry particles were obtained by spray drying using SD Basic, LabPlant with 1.0 mm nozzle diameter at inlet temperature 100°C, flow rate 5 ml/min and pressure 2 mBar.

Particle morphology and size evaluation

The size, shape and surface morphology of the particles were observed using Scanning Electron Microscopy (SEM). Particles were embedded in a holder made of aluminum, and coated with gold palladium prior to analysis. Pictures were taken at various magnifications at 20.00 kV

Fourier Transform Infrared (FT-IR)

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

Differential Thermal Analysis

Thermal analysis of the sample was conducted with Differential Thermal Analyzer (DTA FP-65 P-900 Thermal, Mettler Toledo, USA). About 5 mg of particles were placed in a crucible pan, sealed and observed for its thermogram. The thermogram was recorded at temperature 50 to 250°C with a heating rate 10°C/min.

X-ray Diffractometry

X-ray diffraction analysis was conducted by Phillips X'Pert X-ray diffractometer to evaluate sample's crystallinity. The light source employed was Kα Cu Ni. The voltage and the current were set at 40 kV and 40 mA. Samples were analyzed at 2Ø and angle between 5-40º. The diffractogram of the andrographolide-carboxymethyl chitosan particles was compared with diffractogram of andrographolide substance.

Drug Content

The andrographolide content in nanoparticle was determined by High Performance Liquid Chromatography (HPLC) Agilent 1100 Series using reverse phase with a mobile phase consist of methanol - orthophosphoric acid (50:50, pH 3) at a wavelength of 228 nm. 5.0 mg particles weighed accurately was dissolved in 10.0 ml of methanol, filtered through 0.2 µm membrane filter and then 5 ml sample was injected into the HPLC column. The assay is done in triplicate.

The drug content was calculated using the equations below:

$$\%\ \text{Drug content} = \frac{\text{drug amount}}{\text{particle weight}} x\ 100\%$$

***In vitro* release study**

In vitro release study was performed in 50 ml 0.1% w/v sodium lauryl sulfate (SLS) media at 37 ± 0.5°C, 120 rpm using water bath shaker. SLS was used to improve the dissolution of andrographolide.[13] Andrographolide–carboxymethyl chitosan particles equivalent to 2 mg of andrographolide was weighed accurately and put into the media. 1.0 ml sample was taken at a predetermined time for 3 hours and the same volume of media was added after sampling. Andrographolide was also evaluated as control. The samples were analyzed using HPLC as mentioned above. This evaluation was done triplicate.

***In vivo* antimalarial activity test**

In vivo antimalarial activity test was conducted according to a method of Peter method, The 4-day suppressive test of blood schizonticidal action.[14] 1.5 - 2 months age, male mice strain Balb/C weighing 20-30 gram supplied by the Department of Parasitology, Faculty of Medicine, University of Brawijaya, Malang. All mice were acclimatized in animal house and were fed with standard diet and water *ad libitum*. The use of animal in this study was approved by the Animal Care and Use Committee (ACUC) of Veterinary Faculty, Airlangga University (591-KE). All mice were injected intraperitoneally with 200 ml *Plasmodium berghei* infected mice's blood which contained approximately 10^5 parasitized erythrocytes. Sixteen mice were used and were divided into four groups. Group 1 and group 2 were orally treated twice daily for four days with andrographolide suspended in carboxymethyl chitosan solution, andrographolide-carboxymethyl chitosan particles suspended in water respectively with the dose equivalent to 12.5 mg andrographolide/kg. Group 3 was received carboxymethyl chitosan solution and group 4 as the untreated/control group was received water. During 6 days, tail blood was withdrawn and

parasitaemia was monitored by examining Giemsa-stained smear thin blood using an optical microscope. Parasitaemia was calculated by the equation:

$$\%\ \text{parasitaemia} = \frac{\text{number of parasitized erythrocytes}}{\text{number of erythrocytes}} \text{x } 100\%$$

Percentage of parasitaemia inhibition was calculated on day 5 using equation below.

$$\%\ \text{inhibition} = 100\% - \frac{\%\ \text{parasitaemia growth of treated group}}{\%\ \text{parasitaemia growth of untreated group}} \text{x } 100\%$$

RESULT and DISCUSSION

Figure 1 showed the morphology of andrographolide-carboxymethyl chitosan nanoparticles and non-crosslinked andrographolide-carboxymethyl chitosan. In crosslinked andrographolide-carboxymethyl chitosan micrograph, it was shown nonspherical with hollow shape while the non-crosslinked andrographolide-carboxymethyl chitosan produced more spherical and smooth particles. The andrographolide crystal was observed in crosslinked particles. It indicated that andrographolide was solidified thus hindered the formation of spherical and smooth particles shape. Crosslinked andrographolide-carboxymethyl chitosan nanoparticles had the range size of 600 nm to 3000 nm since the non-crosslinked andrographolide-carboxymethyl chitosan particles size within the range of 500 nm to 2500 nm.

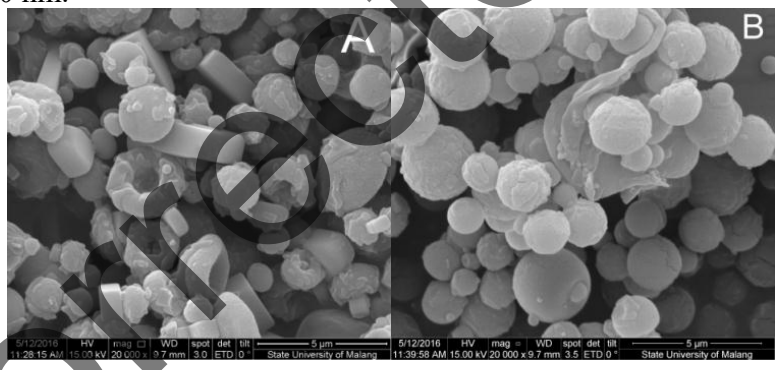

Figure 1. SEM of crosslinked andrographolide- chitosan particles (A) and non crosslinked andrographolide- chitosan particles (B) (magnification 20000 x)

Differential Thermal Analysis (DTA)

From DTA thermogram in Figure 2, it was identified that andrographolide had a melting range 231.6ºC with a sharp endothermic peak, while non-crosslinked andrographolide-carboxymethyl chitosan particles had a sharp endothermic peak with melting point 162.9°C. Thermogram of andrographolide-carboxymethyl chitosan nanoparticles shifted to the lower melting point of 125.7°C with wide endothermic peak compared to andrographolide and the non-crosslinked andrographolide-carboxymethyl chitosan particles. It indicated that the formation of crosslinked particles resulted in lower ordered

structural molecule pattern compared to the non-crosslinked particles and andrographolide substance.

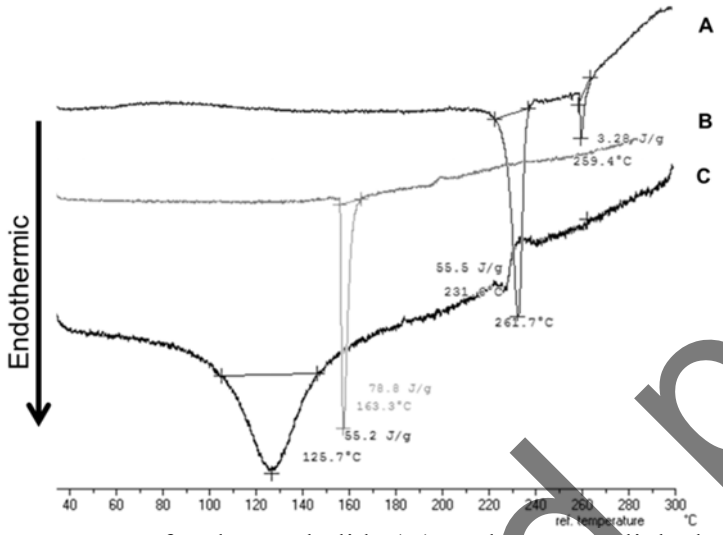

Figure 2. DTA thermogram of andrographolide (A), and non-crosslinked andrographolide-chitosan particles (B), crosslinked andrographolide-chitosan particles (C)

X-ray Diffractrometry

As shown in Figure 3, the diffractogram of andrographolide indicated crystalline peaks with high intensity on 2Ø 9.83°, 14.81°, 15.69°, 15.85°. $CaCl_2$ itself had diffraction peak at 14.74° 2Ø. In diffractogram of andrographolide-carboxymethyl chitosan physical mixture, several crystalline peaks with low intensity at 12.14°, 15.77° and 18.55° were detected.

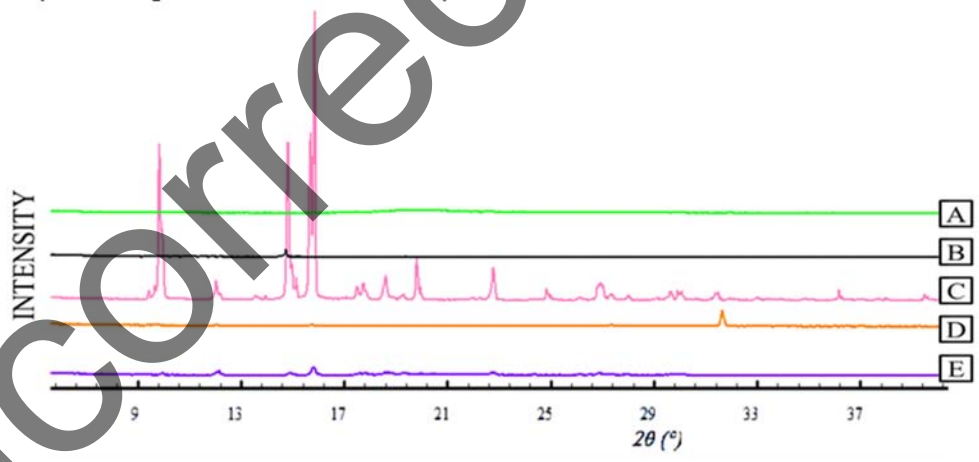

Figure 3. X-ray diffractogram of (A) carboxymethyl chitosan, (B) $CaCl_2$, (C) andrographolide (D) crosslinked andrographolide- chitosan particles, dan (E) physical mixture of andrographolide-chitosan

Meanwhile, the diffractogram andrographolide-carboxymethyl chitosan nanoparticles showed that diffraction peaks of andrographolide, carboxymethyl chitosan, and $CaCl_2$ disappeared and but new crystalline peaks at 31.63° 2Ø appeared. These results showed

that the formation of crosslinked particles of andrographolide-carboxymethyl chitosan prepared by ionic gelation-spray drying has lower crystallinity compared to andrographolide itself.

Drug content

From drug content evaluation with HPLC method, andrographolide content in the particles was 12.09 ± 0.26 %. The result was further used to calculate the amount of andrographolide-carboxymethyl chitosan for *in vitro* release test and *in vivo* antimalarial activity test.

***In vitro* release study**

The *in vitro* release test was performed in 0.1% SLS to facilitate drug dissolution. The result demonstrated that andrographolide dissolved in 15 minutes from nanoparticle systems (69.06%) was greater compared to andrographolide which not dissolved yet. After 30 minutes, andrographolide dissolved from the nanoparticles was up to 10 times higher than andrographolide substance.

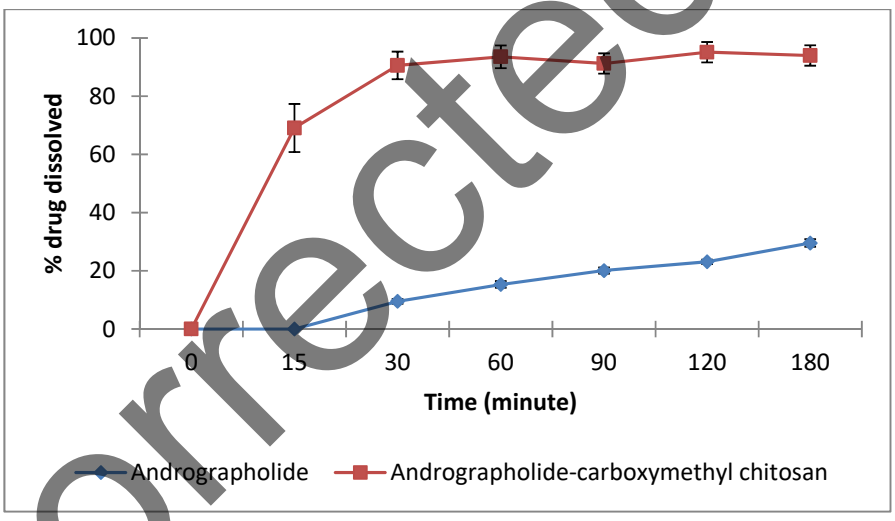

Figure 4. *In vitro* release profile of andrographolide-carboxymethyl chitosan particles and andrographolide in 0.1 % SLS media at 37 ± 0.5°C. (n=3)

The slope that indicating the release rate of andrographolide from carboxymethyl particles and andrografolide substance were 12.7158±0.8054% dissolved/min$^{1/2}$ and 2.0221±0.2702% dissolved/min$^{1/2}$ respectively. The result indicated that the formation of andrographolide-carboxymethyl chitosan could increase the release rate of andrographolide 6.3 times compare to andrographolide itself. This was due to the formation of particles prepared by ionic gelation method - spray drying leads to the changes in the crystallinity of the drug as well as has been shown by the results of the DTA thermogram and X-ray diffractogram in Figure 2 and Figure 3. The entrapment of andrographolide into the cross-linked carboxymethyl chitosan continued with fast

solidification of the particles during spray drying process caused inhibition of crystal growth could result in an amorphous form and crystal size reduction of andrographolide. The changes of crystallinity structure into the amorphous form and reduction of particle size of poorly soluble drug would be advantageous since it will enhance the solubility and then its bioavailability. [15,16]

In vivo antimalarial activity test

Figure 5 showed that parasitaemia growth occurred during the evaluation at all groups. The treated groups led to a slow growth of parasitemia, since the untreated/control group had rapid growth parasiteamia. Antimalarial activity test results presented in Figure 5 and Figure 6 revealed that in all treated groups the increasing number of *Plasmodium berghei* infected erythrocytes (parasitaemia) were lower than the control/untreated group.

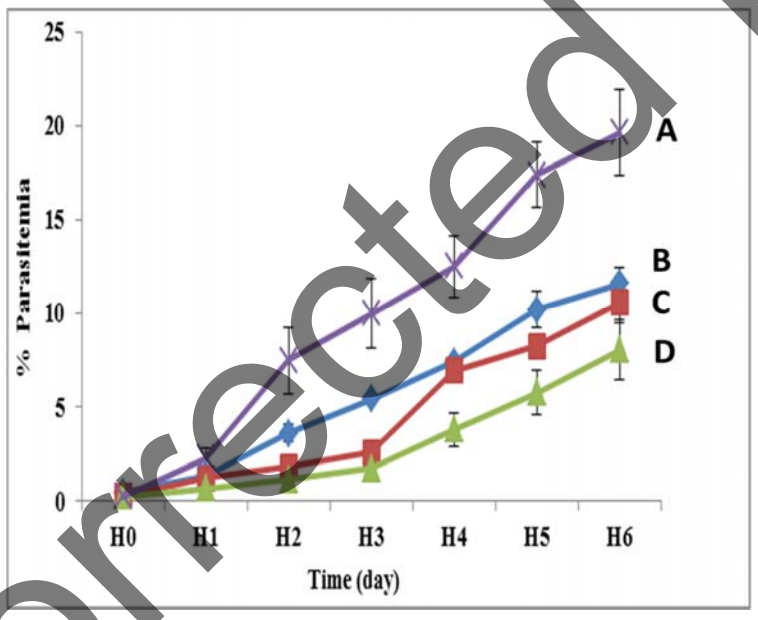

Figure 5. Parasitaemia growth curve of *Plasmodium berghei* infected mice of (A) control group, (B) andrographolide treated group, (C) carboxymethyl chitosan treated group and (D) andrographolide-carboxymethyl chitosan particles treated group

The growth inhibition of parasitemia on day five of andrographolide system-carboxymethyl chitosan nanoparticles treated group was 71.27 ± 6.83%, higher compared to andrographolide treated group (43.30 ± 1.83%) and carboxymethyl chitosan treated group (45.48 ± 3.71%). The formation of andrographolide nanoparticle with carboxymethyl chitosan has changed the physical state of andrographolide which lowered its melting point and degree of crystallinity as shown in Figure 2 and Figure 3. Those result will improve the dissolution of andrographolide and further will provide a favorable effect on its activity. The parasitemia growth inhibition of the andrographolide-carboxymethyl chitosan particles

increased 1.65 times compared to andrographolide and significantly different statistically (Table 1).

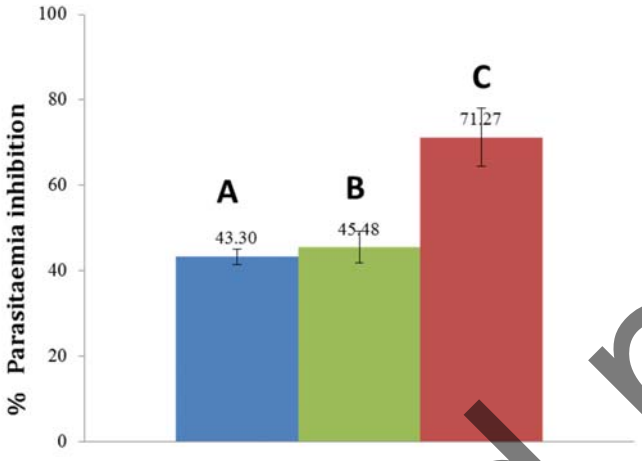

Figure 6. Histogram of parasitaemia inhibition percentage of *Plasmodium berghei* infected mice of (A) andrographolide treated group, (B) carboxymethyl chitosan treated group and (C) andrographolide-carboxymethyl chitosan particles treated group

Tabel 1. One-way Anova (p = 0.05) to determine the effect of andrographolide, carboxymethyl chitosan and andrographolide-carboxymethyl chitosan particles on parasitaemia inhibition of *Plasmodium berghei* infected mice

Group	N	%parasitaemia inhibition ± SD	Anova	
			Result	Conclusion
Andrographolide treated group	4	43,30 ± 1,83[a]	F = 11.373 p = 0.002	Significantly different
Carboxymethyl chitosan treated group	4	45,48 ± 3,71[a]		
Andrographolide-carboxymethyl chitosan particles treated group	4	71,27 ± 6,83[b]		

Note: [a,b] signs refer to no difference between the groups. N=sample number

CONCLUSION

The formation system andrographolide-carboxymethyl chitosan nanoparticles affected the physical characteristics of andrographolide. The crystallinity decrease of andrographolide resulted in a lower melting point of andrographolide. Such changes had a positive impact to the drug dissolution and then its activity. The release rate of andrographolide from carboxymethyl chitosan nanoparticles increased up to 6.3 times and *in vivo* antimalarial activity in *Plasmodium berghei* infected mice significantly enhanced up to 1.65 times compared to the andrographolide substance.

ACKNOWLEDGEMENT

This research was financially supported by Research Grant of Faculty of Pharmacy, Airlangga University, 2016.

CONFLICT OF INTEREST

Declared none

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