Research Article

Centella asiatica Extract Loaded BSA Nanoparticles Using the Organic and Conventional *C. asiatica* to Improve Bioavailability Activity and Drug Delivery System

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Abstract
Centella asiatica (CA) extracts have been described for their high phytochemical contents, especially phenolic compounds. Active extracts also showed effectively potential *in vitro* but not *in vivo* experiments due to their poor lipid solubility or inappropraietd molecular weight, which resulted in poor bioavailability. This study, the nanoencapsulation process is applied to enhance bioavailability, stability and bioactivity of CA extracts. BSA (Bovine serum albumin) nanoparticles containing phenolic extracts of CA were synthesized by an adapted desolvation method at the ratio between CA extract: BSA at 1:2, 1:3 and 1:4. The entrapment efficiency, loading efficiency, solubility and stability are used to test the efficiency of the nanoparticles. The in vitro released kinetic is monitored for 6-hour period in both artificial gastric buffer at pH 2.0 and intestinal juice buffer at pH 7.4. The result showed that the different ratio of the CA concentrations to BSA nanoparticles had no significant effect to its bioavailability ($p < 0.05$). On the other hand, the types of extraction solvents including ethanol, chloroform and hexane significantly affected the level of bioavailability ($p < 0.05$). Especially, the ethanol extracts loaded in BSA at ratio of 1:2 showed the best result, and it was the most economical way due to less consumption of BSA nanoparticle was used. The study of CA extracts loaded in BSA nanoparticles here demonstated the improvement of bioavailability and drug delivery system.

Keywords: *Centella asiatica*, BSA-nanoparticles, Bioavailability, Organic, *in vitro* released kinetic

1 Introduction

Centella asiatica (CA) is top five of Thailand Champion Herbal Product (TCHP) needed in the world market and consisted of high efficiency, which could be applied in pharmaceutical, cosmetic and food industries. It has been already investigated for its bioavailability activity, antimicrobial activity, antioxidant activity,

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anti-inflammatory activity, wound healing activity and anticancer activity [1], [2]. The major bioactive compounds of CA extracts are triterpene glycosides (saponins), such as asiaticoside and madecassoside, and aglycones (sapogenins), including asiatic acid and madecassic acid [3], [4].

Although CA extracts showed their biological potential as active compounds in vitro but they possessed less or no activity *in vivo* due to their poor solubility in aqueous solution and improper molecular size. It is not easy for water soluble biological active compounds to across the cell membrane, which has hydrophobic characteristic, to the cells of both human and pathogenic microorganisms [5], [6]. These properties of CA extracts resulted in poor absorption, slow delivery, poor dosing and poor bioavailability.

The nanotechnology is one of the challenging technology to improve herbal extract's bioavailability by improving drug delivery to the targets and enhancing the stability of active chemicals for drug formulation. This technology could also reduce operational cost of extraction to obtain the pure biological active compounds. The formulations of herbal extracts with nanoparticles could lead to enhancement of the activity of plant extracts, promotion of the constant release of active compounds, reduction of the required dose and decreasing of side effects [7]. According to various studies, nanoparticles technique is suitable to improve bioavailability and drug delivery systems via oral administration [8]. The aim of this study was to improve the bioavailability and drug delivery system of CA by formulation with BSA-nanoparticles and to compare the organic and conventional raw materials to increase product's value in the market.

2 Materials and Methods

2.1 *Preparation of plant materials*

CA plant material was purchased from local markets in Bangkok, Thailand. The aerial part of CA was separated from other parts by manually cutting. Fresh CA samples were washed with tap water and chopped into small pieces. Then, plant materials were dried in hot air oven at 45°C until the constant weights were achieved. The dried samples were finely ground into powder and kept at 4°C before used [9].

2.2 *Preparation of CA crude extract*

CA powders obtained from commercial farming and organic farming were subjected to solvent extraction with 95% ethanol, chloroform and hexane using loading ratio between powder and solvent volume at 10% w/v [10]. The mixtures were macerated at room temperature, and the mixing was done at 120 rpm, for 48 h. Then, the liquid fractions were collected by filtration with Whatman filter paper No.4. The crude extracts were concentrated by using rotary evaporators (BUCHSI ROTAVAPOR R-205) at 50°C and the concentrated extracts were kept at –20°C until use. The CA crude extracts were further subjected for preparation of CA-BSA nanoparticles.

2.3 *Preparation of CA extract-loaded BSA nanoparticles*

CA extract-loaded BSA nanoparticles was prepared by using the desolvation method as described in previous study [11], [12] The 100 mg of BSA were dissolved in 1 mL of 10 mM sodium chloride solution. Then, 8.0 mL of ethanol was added dropwise into the BSA solution under magnetic stirring at 400 rpm. Subsequently, the prepared BSA nanoparticles were cross-linked with 0.2% glutaraldehyde (GA). Then, CA crude extract was added into the BSA solution for 24 h with different loading ratio of CA crude extracted to BSA at 1:2, 1:3 and 1:4 to prepare CA extract-loaded BSA nanoparticles. The particles were collected by using centrifugation and washed with distilled water. The collected nanoparticles were resuspended and dispersed in 2% mannitol solution, then freeze-dried at –40°C for 24 h. The dried nanoparticle powders were kept at room temperature until use.

2.4 *Preparation of CA extract-loaded BSA nanoparticles*

CA crude extract was analyzed by scanning of absorbance spectrum to find the best the absorbance wavelength (λmax) at which the absorbance value was the highest by using UV-vis spectrophotometer (CamSpec Model M508). The 2 mg of CA extract-loaded BSA nanoparticles were dissolved in 1 mL methanol and gently shaken for 24 h at 37°C to completely dissolve CA to methanol. Then, the dissolved

samples were centrifuged at 12,000 rpm for 10 min to separate the undissolved portions of nanoparticles. The supernatant was collected and its optical density (OD) at λmax was measured. All measurements were done in triplicate with three replications independently. The amount of CA crude extract that was entrapped and loaded in nanoparticle was calculated and expressed as entrapment efficiency and loading efficiency as follows [13], [14]

$$Entrapment\ efficiency(\%) = \frac{amount\ of\ crude\ extract\ in\ nanopaticles}{amount\ of\ total\ CA\ crude\ extract} \times 100$$

$$Loading\ efficiency(\%) = \frac{amount\ of\ crude\ extract\ in\ nanopaticles}{amount\ of\ nanopaticles} \times 100$$

2.5 *Solubility and stability tests*

The solubility of CA crude extract in tested solutions was separately determined and compared between before and after the encapsulation process to calculate saturation solubility of CA crude extract. CA crude extract and CA extract-loaded BSA nanoparticles solubility were tested by resuspending them in 1 mL of distilled water and stirred the mixture at 200 rpm, 37°C. After 24 h, undissolved samples were separated by filtration through a 0.22 μm Millipore membrane cassette equipped with vacuum pump. Filtrate was diluted with distilled water appropriately, and the optical density (OD) at λmax of each sample was measured by a UV-vis spectrophotometer.

The stability of CA crude extract was evaluated by adding 1 mg/mL of CA extract-loaded BSA nanoparticles in 0.01 M phosphate buffer solution, pH 7.4. The samples were mixed with magnetic stirrer at 200 rpm, 37°C, for 24 h. At different sampling time points (0, 0.5, 1, 2, 3, 4, 5 and 6 h), the mixture was sampled and the optical density (OD) at λmax was measured [14], [15]. All measurements were done in triplicate with three replications independently. The stability of CBNP was determined and calculated as follows

$$Percentage\ reducttion\ of\ CA\ concentration = (100)\left(\frac{C_0 - C_t}{C_0}\right)$$

Where C_0 is OD at λmax value of the sample at the time zero

C_t is OD at λmax value of the sample at sampling time point (0, 0.5, 1, 2, 3, 4, 5, and 6 h)

2.6 *Release kinetic in vitro*

Monitoring of released kinetic of CA extract in nanoparticle was conducted as described in previous studies with minor modification [16]–[18]. The release experiment of CA crude extract from CA-BSA nanoparticles was performed by dissolving 20 mg of CA-BSA nanoparticles in 15 mL of artificial gastric juice (containing 0.01 M of PBS, pH 2.0) and intestinal juice without enzymes (containing 0.01 M of PBS, pH 7.4). The mixture was incubated in shaking incubator at 37°C, with shaking speed at 200 rpm. At targeted time points (0, 1, 2, 3, 4, 5, and 6 h), each mixture was sampled and collected by centrifugation at 3,000 rpm for 10 min. The pellet was resuspended in 100 μL of methanol again to determine the remaining amount of CA crude extract in nanoparticle, and the optical density (OD) at λmax of resuspended pellet was measured. All measurements were done in triplicate and three replications independently, then release rate of CA extract of each tested condition was expressed in drug release models.

2.7 *Statistical analysis and Experimental design*

All experiments were conducted in three replications and statistical analysis was conducted using ANOVA with Duncan's multiple range tests ($p < 0.05$) by SAS software version 9.4.

3 Results and Discussion

3.1 *Entrapment efficiency*

One of the important parameter to determine the drug efficiency is the quantity of bioactive compounds loaded or entrapped in carriers. Entrapment efficiency is usually selected as a parameter to be monitored in study of drug delivery of active compounds in a certain system of drug carrier [19]. For this experiment, the tested samples are the mixtures of CA crude extract, as an active compound, and BSA, as a carrier. Different proportions between CA crude extract and BSA were set to be ratio at 1:2, 1:3 and 1:4 based on dried weight. The experiment to determine the percentage of entrapment efficiency of CA crude extract in

BSA nanoparticle were designed and conducted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version 9.4.

In this work, three extraction solvents, including ethanol, chloroform and hexane, and two different sources of raw materials of CA, including commercial farming and organic farming were selected to be the tested parameters. The entrapment efficiency of CA crude extract-loaded BSA nanoparticles in each condition was evaluated and calculated based on the OD at λmax (Table 1). Based on the result, the entrapment efficiency of CA-BSA nanoparticles mixture obtained from 3 types of solvents at ratio 1:4 was significantly higher than that of ratio 1:2 and 1:3 ($p < 0.05$). The higher entrapment efficiency at ratio 1:4 could be due to the more available numbers of carriers, i.e. BSA, therefore more numbers of CA active compounds could be captured in the system. Additionally, the entrapment efficiencies of CA-BSA nanoparticles prepared from CA raw materials obtained from conventional and organic farms had no difference ($p < 0.05$), suggesting that the active compounds from both sources were indifferently captured by BSA nanoparticles.

Table 1: Entrapment efficientcy of CA crude extract loaded BSA nanoparticles

Sample		Entrapment Efficientcy (%)	
Solvent Extraction	**Ratio of Crude:BSA**	**Conventional Farm**	**Organic Farm**
Ethanol	1:2	46.26 ± 0.97^{E}	43.23 ± 0.37^{E}
	1:3	70.91 ± 2.31^{B}	66.40 ± 1.12^{BC}
	1:4	93.12 ± 0.96^{A}	90.60 ± 0.31^{A}
Chloroform	1:2	43.22 ± 3.29^{E}	43.18 ± 0.36^{E}
	1:3	56.21 ± 2.48^{D}	64.72 ± 0.68^{BC}
	1:4	94.00 ± 4.51^{A}	89.10 ± 0.46^{A}
Hexane	1:2	42.99 ± 1.99^{E}	39.19 ± 2.04^{E}
	1:3	67.86 ± 1.94^{BC}	63.26 ± 4.44^{BC}
	1:4	87.82 ± 3.31^{A}	89.23 ± 2.74^{A}

Note: Different superscript within a column showed significant different at $p < 0.05$

3.2 *Loading efficiency*

Loading efficiency is a parameters used to represent the quantity or amount of CA extracted bioactive compounds loaded into the BSA carrier to the total of BSA added [19]. In this section, three different ratio between CA crude extract and BSA, three types of solvents, and two sources of CA raw materials were compared (Table 2). Based on statistic analysis, there were no significant difference in loading efficiency in all ratio of CA extract loaded BSA nanoparticles among all extraction solvents ($p > 0.05$). In addition, there were also no significant difference between conventional and organic farms ($p > 0.05$) (Table 2). Moreover, results showed that almost 40% of the CA bioactive compounds were loaded in the BSA nanoparticles. However, the improvement of entrapment efficiency could be done by extending the mixing time for carrier particles to entrap more active compounds and adjusting the addition time of BSA into the system. The others factors, including pH and temperature, were also demonstrated previously to influence in entrapment efficiency and loading capacity of active compounds loaded other carrier, i.e. poly(lactic-co-glycolic acid) nanoparticles [20].

Table 2: Loading efficiency of CA crude extract loaded BSA Nanoparticles

Sample		Entrapment Efficientcy (%)	
Solvent Extraction	**Ratio of Crude:BSA**	**Conventional Farm**	**Organic Farm**
Ethanol	1:2	39.32 ± 0.82^{A}	36.74 ± 0.31^{ABC}
	1:3	39.00 ± 1.27^{AB}	36.52 ± 0.62^{ABC}
	1:4	37.34 ± 0.38^{ABC}	$36.24 \pm .013^{ABC}$
Chloroform	1:2	36.74 ± 2.80^{ABC}	36.71 ± 0.31^{ABC}
	1:3	30.92 ± 1.37^{D}	35.60 ± 0.37^{ABCD}
	1:4	37.60 ± 1.80^{ABC}	$35.64 \pm .018^{ABCD}$
Hexane	1:2	36.54 ± 1.69^{ABC}	33.31 ± 1.74^{CD}
	1:3	37.32 ± 1.07^{ABC}	34.79 ± 2.44^{CD}
	1:4	35.13 ± 1.32^{ABCD}	35.69 ± 1.10^{ABCD}

Note: Different superscript within a column showed significant different at $p < 0.05$

3.3 *Solubility*

The evaluation of solubility of CA crude extract was performed in distilled water under controlled condition at pH 7.0, 37°C for 24 h (Table 3). The result showed that CA-BSA nanoparticles were barely dissolved in water when compared to CA crude extract. Moreover, there were significant difference between CA crude extract and CA extract loaded in BSA nanoparticle ($p < 0.05$). Therefore, BSA nanoparticles prepared from desolvation methods can be used to protect CA crude extract from undesired condition and deliver hydrophilic bioactive compounds to attach and penetrate through cell membranes [21] of human and pathogenic

bacteria with slow release rate, in which their cell membranes allow only hydrophobic compounds to access [22], [23].

Table 3: Solubility of CA crude extract loaded BSA nanoparticles and CA crude extract

Sample		Entrapment Efficientcy (%)	
Solvent Extraction	**Ratio of Crude:BSA**	**Conventional Farm**	**Organic Farm**
Ethanol	1:2	202.62 ± 7.17^{EF}	209.30 ± 5.39^{DE}
	1:3	176.08 ± 2.69^{FGH}	196.42 ± 7.84^{EF}
	1:4	159.37 ± 4.23^{H}	200.71 ± 6.92^{EF}
Chloroform	1:2	220.46 ± 2.35^{CD}	224.66 ± 2.21^{CD}
	1:3	169.18 ± 6.22^{FGH}	211.55 ± 2.69^{CDE}
	1:4	192.43 ± 6.49^{EFG}	194.06 ± 2.64^{EFG}
Hexane	1:2	167.75 ± 6.42^{GH}	183.88 ± 8.38^{FG}
	1:3	173.37 ± 2.80^{FGH}	166.34 ± 5.16^{H}
	1:4	167.75 ± 5.64^{H}	173.07 ± 8.39^{FGH}
CA Crude Extracts			
Ethanol		374.21 ± 4.49^{A}	298.98 ± 5.53^{B}
Chloroform		245.40 ± 9.10^{C}	234.68 ± 1.38^{C}
Hexane		254.13 ± 5.28^{C}	234.08 ± 4.73^{C}

Note: Different superscript within a column showed significant different at $p < 0.05$

3.4 *Stability*

To determine the solubility of CA crude extracts before and after the encapsulation process, CA crude extracts and CA extract-loaded BSA nanoparticles were dissolved in water. The results were interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version 9.4. The remaining CA crude extracts were monitored in different sampling time points by measurement of OD at λmax (Figure 1). In this experiment, the stability of CA extract-loaded BSA nanoparticles was tested in the PBS solution at pH 7.4 for 6 hour-long period. Similarly to the previous section, CA extract-loaded BSA nanoparticles obtained from different preparations, including 3 types of solvents, 3 ratios of CA extract and BSA, and 2 sources of raw materials were challenged in this stability test.

The results showed that in all samples the remaining CA crude extract from one to six hour periods were in ranged of 40–60% of each BSA nanoparticle sample and the trend was really constant at the same level of amount (Figure 1). This consistency of CA crude extract amounts in the tested system indicated that the

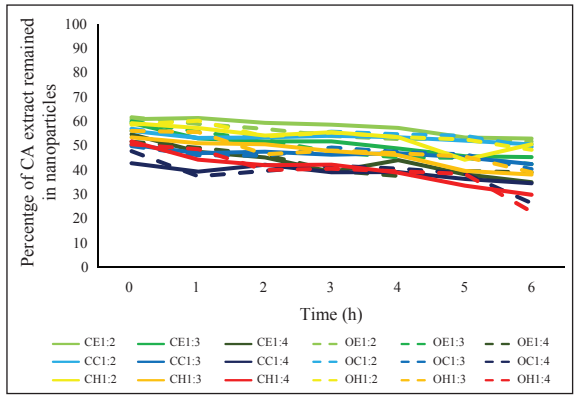

Figure 1: Stability of CA loaded- BSA nanoparticles in PBS, pH7.4 at 37°C. CE = Conventional CA ethanol extract, CC = Conventional CA chloroform extract, CH = Conventional CA hexane extract, CE = Organic CA ethanol extract, CC = Organic CA chloroform extract, CH = Organic CA hexane extract, 1:2, 1:3 and 1:4 represented ratio between crude : BSA.

nano-encapsulation process assisted the stability of the CA crude extract by protecting it from hydrolysis and biotransformation. This experiment showed high potential of CA extract-loaded BSA nanoparticles for prolonging the shelf-life of the extracted herbal product [24]. Furthermore, this BSA nano-encapsulation improved active compounds hydrophobicity and therefore increased the potential to transport across cell membranes of targeted organisms [25]. Moreover, from each extraction solvent, at ratio 1:2 showed the highest stability of CA crude extract, which was a benefit in economical aspects due to the reduction of the usage of the BSA nanoparticle.

3.5 *Release kinetic in vitro at pH 2.0 and pH 7.4*

For study of release kinetic in vitro, CA extract-loaded BSA nanoparticles with 3 different extraction solvents and 2 different sources of raw materials were tested in the artificial gastric juice (0.01 M PBS pH 2.0) and intestinal juice without enzymes (0.01 M PBS pH 7.4) in order to imitate the environment in human stomach and intestine, respectively (Figures 2 and 3). The release rate of CA extract-loaded BSA nanoparticles with the same ratio in the simulated gastric juice (ranging between ~10–20%, Figure 2) tended to be higher than intestinal juice (ranging between ~4–8%, Figure 3).

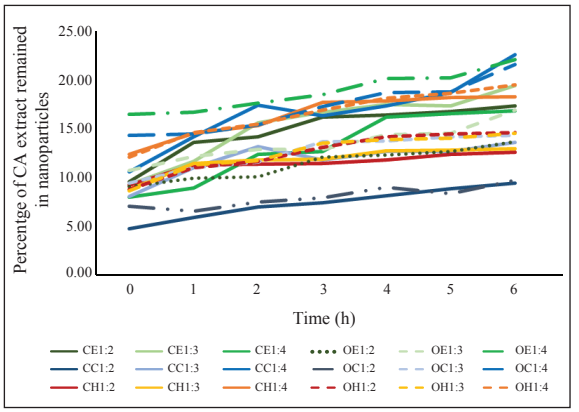

Figure 2: Release rate of CA- BSA nanoparticles *in vitro* in artificial gastric juice at 37°C. CE = Conventional CA ethanol extract, CC = Conventional CA chloroform extract, CH = Conventional CA hexane extract, CE = Organic CA ethanol extract, CC = Organic CA chloroform extract, CH = Organic CA hexane extract, 1:2, 1:3 and 1:4 represented ratio between crude : BSA.

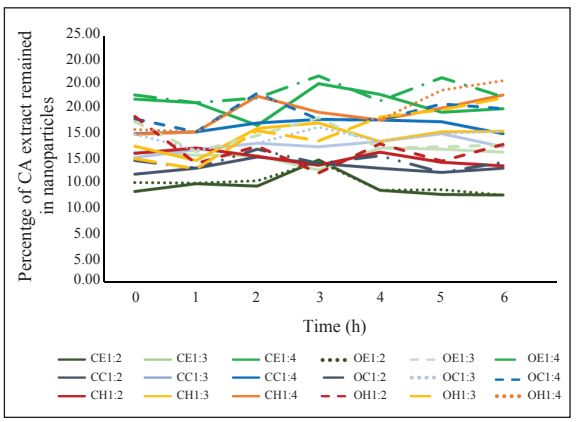

Figure 3: Release rate of CA-BSA nanoparticles *in vitro* in artificial intestinal juice at 37°C. CE = Conventional CA ethanol extract, CC = Conventional CA chloroform extract, CH = Conventional CA hexane extract, CE = Organic CA ethanol extract, CC = Organic CA chloroform extract, CH = Organic CA hexane extract, 1:2, 1:3 and 1:4 represented ratio between crude : BSA.

The reason was because the acidic condition in gastric juice at pH 2.0 could denatured the protein structure, i.e. BSA [26], which caused the protein structure to unfold and then bioactive compounds were released at higher rate comparing to the release rate of CA extract-loaded BSA nanoparticles in PBS pH 7.4 (Figure 3). Furthermore, as the time increased for CA extract-loaded BSA nanoparticles in the PBS solution, the higher released rate of CA extract was observed. At pH 7.4, the CA extract-loaded BSA nanoparticles became more stable during 6 h in the PBS solution (Figure 3). Moreover, according to Suwantong *et al*., Yu *et al*. [27], [28], the albumin protein was demonstrated to be stable in the pH range from 4.0 to 8.0, and it could be heated at 60°C up to 10 h without any deleterious effects.

4 Conclusions

The nanoparticles technology was demonstrated to be used to improve the bioavailability of CA crude extract. Based on the experiment data, there was no significant difference between the ratio of CA to BSA nanoparticle (1:2, 1:3, and 1:4) and between conventional and organic CA in the term of the CA extract-loaded in the BSA nanoparticle ($p < 0.05$). While, the extraction solvent types as ethanol, chloroform and hexane were significantly different ($p < 0.05$). The CA ethanolic extract-loaded in BSA nanoparticle at ratio 1:2 will be suggested for further applications such as in food supplement, cosmetic or personal care in economic aspects due to less required amount of BSA nanoparticle.

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