Study on the effect of microemulsion formulation on oral bioavailability of *Morus alba* stem extract using *in vitro* lipolysis model

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Abstract

Microemulsions, which have been reported to enhance oral bioavailability of various compounds, were previously developed to incorporate *Morus alba* stem extract (MSE). Their potential abilities to improve oral bioavailability of MSE were aimed to investigate in this study using *in vitro* lipolysis model. Firstly, four microemulsions formulations (F1-F4) investigated in this study were prepared. They consisted of 10% oil phase, 70% surfactant mixture (Smix) and 20% aqueous phase. The oil phase of all formulations was caprylic/capric triglyceride. The Smix of F1, F2, F3 and F4 were the mixture of PEG-8 caprylic/capric triglyceride (surfactant, S) and polyglyceryl-3 diisosterate (co-surfactant, CoS) at weight ratios of 2:1, 3:1, 4:1 and 2:1, respectively. The aqueous phase of F1-F3 was water while the aqueous phase of F4 was water mixed with propylene glycol (PG) at ratio 1:1. Their characteristics including viscosity, particle size and cloud point were evaluated. Their physical stability was determined by cooling-heating cycling test. All formulations have similar viscosity and particle size. However, cloud point temperature of these formulations was different and F4 showed the highest cloud point. In addition, no significant change in physical characteristics was observed after stability test. Thus F4 was selected to incorporate MSE and subjected to *in vitro* intestinal lipolysis study. MSE dissolved in 50% PG solution was used as a control. F4 consisting of 10%caprylic/capric triglyceride/70% PEG-8 caprylic/capric glycerides mixed with polyglyceryl-3 diisostearate (3:1)/10%water/10%PG was shown to enhance the digestion of MSE greater than the PG solution. This study suggests the potential of microemulsion in the enhancement of bioavailability of MSE in *in vivo*. Animal study is suggested to be further performed.

Keywords: Mulberry stem extract, microemulsion, bioavailability, *in vitro* lipolysis model

Introduction

Morus alba L., also known as white mulberry or Mhon, is classified in the genus *Morus* of the family Moraceae. It is widely cultivated under support of the Queen Sirikit Department of Sericulture to direct farmers to mulberry cultivation and silk farming. Our previous study has been investigated the efficacy of *M. alba* stem ethanol extract (MSE) on pain relief in rat model of osteoarthritis (OA) which was induced by anterior cruciate ligament transection (ACLT). The pain-related behavior was determined by measuring hind limb weight bearing (Khunakornvichaya, et al., 2012 & 2016). The results showed that MSE significantly attenuated joint pain in

dose-dependent manner, and it showed comparable effect with glucosamine (Khunakornvichaya, et al., 2012 & 2016). Its effect on inhibition of nitric oxide production in LPS-induced RAW 264.7 macrophage cells was also demonstrated (Soonthornsit et al., 2013). In addition, the antioxidant capacities of MSE were proved by various *in vitro* antioxidant assays (Pham et al., in press). However, we observed that water solubility of the extract was limited. It is generally known that a compound with low water solubility often causes poor absorpyion, resulting in poor oral bioavailability. Therefore, a drug delivery system which can improve solubility and thus enhances oral absorption of the extract was considered essential.

Microemulsion is isotropically dispersion of two immiscible liquids such as oil and water, stabilized by an interfacial film of surfactant and co-surfactant molecules (Eccleston, 1992). It was gaining interest in this study because it has been reported to improve drug solubilization and to keep the drug in solution form as it is traveling along gastro-intestinal tract. In addition, it has been shown to enhance oral bioavailability of various soluble and insoluble drugs such as cefpirom, cefodizim (Mrestani, et al., 2010), cyclosporin A (Gao, et al., 1998), acyclovir (Ghosh, et al., 2006), curcumin (Cui, et al., 2009). However, the potential ability of microemulsions to improve bioavailability of MSE has not been investigated. Therefore, this study was aimed to investigate the ability of microemulsion formulations to potentially enhance oral bioavailability of MSE by using *in vitro* lipolysis model.

Materials and Methods

Materials

HPLC grade acetonitrile was purchased from RCI Labscan (Bangkok, Thailand). 4-bromophenylboronic acid, pancreatin from porcine pancreas, sodium taurodeoxycholate (NaTC), Trizma maleate, L-α-phosphatidylcholine and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Caprylic/capric triglyceride was purchased from Croda Singapore Pte., Ltd. (Singapore). PEG-8 caprylic/capric glycerides and polyglyceryl-3 diisostearate were from Gattefosse (Lyon, France). Calcium chloride dihydrate ($CaCl_2.2H_2O$) and sodium hydroxide were purchased from Merck (Darmstadt, Germany). MSE has been prepared as described by Soonthornsit et al. (2013, 2017).

Formulation of microemulsion containing mulberry stem extract

Caprylic/capric triglyceride, PEG-8 caprylic/capric glycerides and polyglyceryl-3 diisostearate were chosen as oil, surfactant (S) and co-surfactant (CoS), respectively. The microemulsion formulations were prepared using the compositions shown in Table 1. PEG-8 caprylic/capric glycerides was mixed with polyglyceryl-3 diisostearate (Smix) at various weight ratios, i.e. 2:1, 3:1 and 4.1. The effect of PG, a co-solvent, on the characteristics of microemulsions was also investigated. PG was added to the formulation by mixing with water and used as an aqueous phase.

Table 1 – Compositions (%w/w) of microemulsion formulations.

Compositions	F1	F2	F3	F4
Caprylic/capric triglyceride	10	10	10	10
Smix* (2:1)	70	-	-	-
Smix* (3:1)	-	70	-	70
Smix* (4:1)	-	-	70	-
Water	20	20	20	10
Propylene glycol	-	-	-	10

***Smix was PEG-8 caprylic/capric glyceridesmixed with polyglyceryl-3 diisostearate at a specific weight ratio.**

Characterization of microemulsion

Visual inspection

Optically clear single-phase transparent samples were defined as microemulsions.

Polarized light microscopy

Cross-polarized light microscopy was used to confirm the isotropic nature of microemulsions. As isotropic structure of microemulsion does not interfere with the polarized light, thus the field of view under microscopy remains dark (Friberg, 1990).

Viscosity

Viscosity of the microemulsion was measured by using a Brookfield DV-III programmable CP40 cone and plate rheometer (Brookfield Engineering Laboratories Inc., Massachusetts, USA). Measurements were carried out in triplicate at 25°C.

Determination of droplet size in microemulsion

The average droplet size and polydispersity of microemulsion were detected by using Zeta PALS® (Brookhaven instrument, New York, USA) at 25°C.

Cloud point

Cloud point measurement was performed by visual observation in the temperature range 0 to 100°C. The temperature at which microemulsion changed from transparent to turbid mixture was recorded as cloud point.

Short-term stability of microemulsions

The stability of unloaded microemulsions was studied by storing at 4 °C alternate 45 °C for 14 days (cooling-heating cycles). Stability was determined based on the changing of physical characteristics including phase separation, viscosity, droplet size, and cloud point.

Preparation of *M. alba* loaded microemulsion (MSE-ME)

MSE was incorporated in the selected microemulsion at a concentration of 2% by weight. MSE dissolved in 50% PG solution (MSE-PG solution) was used as a control and for comparing with microemulsion.

***In vitro* intestinal lipolysis study**

The absorption of active compound, indicating oral bioavailability, is generally affected by the solubilization capability of the compound in the gastrointestinal tract which can be predicted by *in vitro* lipolysis model. An *in vitro* intestinal lipolysis study was performed using the modified from Yin *et al.* (2009). The tested formulations containing MSE (250 mg) were dispersed in 9 ml of digestive buffer (50 mM Trizma® maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, pH 7.5) containing 5 mM NaTC and 1.25 mM L-α-phosphatidylcholine. The mixtures were incubated at 37°C in a shaking water bath (*Model 1086, GFL Gesellschaft für Labortechnik mbH*, Burgwedel, Germany). The digestion was then initiated by adding 1 ml of pancreatin containing digestive buffer (final lipase concentration: 1000 USP units/ml). The reaction was left under continuous stirring and pH was maintained at 7.5 by adding 0.5 M sodium hydroxide solution. The progress of *in vitro* digestion was indirectly monitored by pH-stat. After 30 min, the digestion was stopped by the addition of 90 µl 0.5 M 4-bromophenylboronic acid solution to inhibit lipase activity. The mixtures were centrifuged at 9,000 rpm for 20 min (Universal Refrigerated Centrifuge, Kubota Corporation, Tokyo, Japan) to separate the aqueous phase and the pellet phase. The aqueous phase was collected and then filtered through 0.45 µm nitrocellulose membrane filters prior to HPLC analysis. The extent of digestion was determined based on the amount of MSE detected in the aqueous

phase compared to the initial amount added and was expressed as a percentage. The amount of MSE in the aqueous phase was detected based on oxyresveratrol by HPLC analysis. The sodium hydroxide consumption during the lipolysis process was also determined.

High Performance Liquid Chromatography (HPLC) analysis of oxyresveratrol

Oxyresveratrol was used as the marker compound for the quantitative HPLC analysis of MSE using the method described by Yhirayha (2013). The stock solutions of oxyresveratrol in 80% ethanol in the concentrations range of 0.0005 to 0.05 mg/ml were prepared. An HPLC system (LC-20AT, Shimadzu, Kyoto, Japan) equipped with a UV-Vis detector (SPD-20A, Shimadzu, Japan), an auto-sampler (SIL-10ADVP, Shimadzu, Kyoto, Japan) and a column oven, was used. Analysis was performed on a C18 boned-silica gel column (Gemini, 5u, 150x4.6 mm, Phenomenex, Torrance, USA) in the isocratic mode. Acetonitrile mixed with 0.05 M phosphate buffer pH 3 (13 ratio) was used as the mobile phase at a flow rate of 1 ml/min. UV detector wavelength of 320 nm with column oven temperature of 30°C were set. The injection volume was 20 µl. Run time was set at 13 min.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was determined using one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons (GraphPad Prism 6.0, GraphPad Software Inc., San Diego, USA). *P*-values less than or equal 0.05 were considered significant.

Results

The characteristics of microemulsions are shown in Table 2. The viscosity of empty microemulsions was ranging from 92 to 125 cP. The average size of all empty microemulsions was in a range of 3.3 to 7.6 nm. The addition of PG in aqueous phase caused an increase in the particle size of F4 microemulsion. Cloud point is important for determining storage stability of formulation, at temperatures significantly higher than the cloud point results in phase separation and instability. As the ratio of S/CoS of the formulation increased (F3>F2>F1), cloud point was getting higher. The microemulsion with PG (F4) showed higher cloud point than that without PG (F2). Following cooling-heating cycling test, a slight change in physical characteristics was observed. As F4 showed the highest cloud point temperature, it was selected to investigate the *in vitro* intestinal lipolysis.

Table 2 Viscosity, particle size and cloud point of unloaded microemulsions after short-term stability test

Formulations	Viscosity (cP)		Particle size (nm)		Cloud point (°C)	
	Before	After	Before	After	Before	After
F1	125.53±0.69	125.10±3.49	3.46±0.05	2.37±0.05	28	32
F2	100.17±0.56	102.57±2.04	3.40±0.08	2.70±0.08	37	31
F3	92.10±0.70	93.10±0.28	3.27±0.12	3.03±0.05	49	38
F4	97.47±0.53	98.87±4.05	7.60±0.12	8.03±0.68	60	60

In vitro lipolysis models, simulating the digestion in the small intestine, has been suggested as a promising tool to determine drug release from lipid-based formulations in a complex process taking place following ingestion of the formulations (Larsen et al., 2011; Xiao et al., 2016). During lipolysis, MSE incorporated in a formulation may remain in the formulation, and be solubilized in the aqueous phase or precipitated out (pellet phase) (Figure 1, right). The percentage digestion was determined from the content of MSE in the aqueous phase. MSE dissolved in PG solution was used as a control. It was observed that the percentage digestion of MSE incorporated in F4 microemulsion greatly enhanced compared to that incorporated in the PG solution (Figure 2). The extent of digestion was also confirmed by the higher sodium hydroxide (0.5 M) consumption for the microemulsion formulation during the lipolysis process (Figure 3).

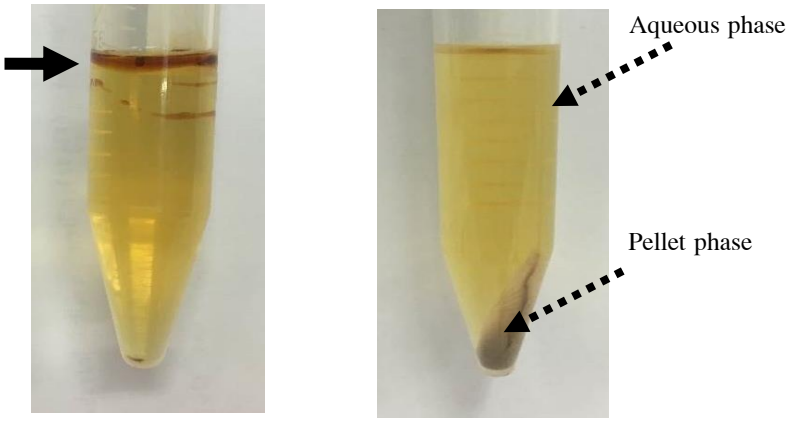


Figure 1 The two phases presented after centrifugation of microemulsion in lipolysis medium in the absence (left) and the presence (right) of pancreatin enzyme.

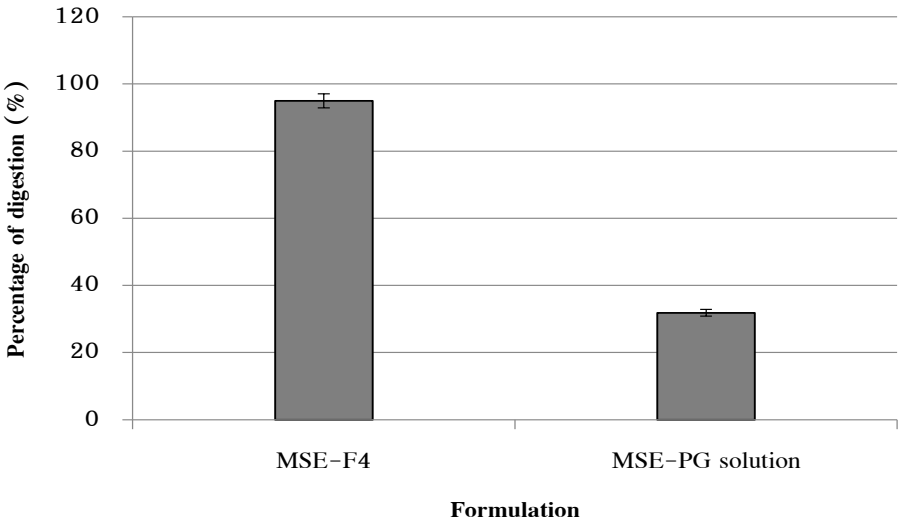
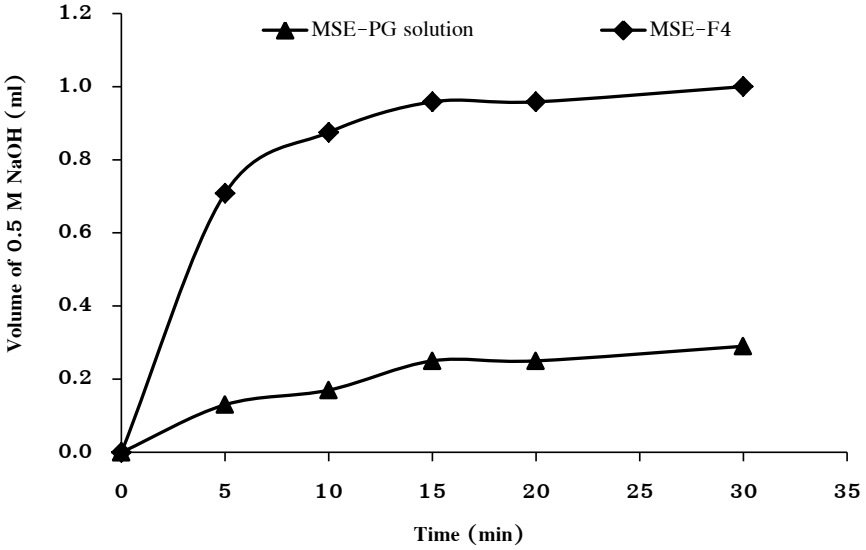


Figure 2 The percentage of *M alba* stem extract in the aqueous phase (i.e. percentage of digestion).



Comparing amoung microemulsion formulations prepared, F4 showed the highest cloud point and thus it was selected for the determination of *in vitro* lipolysis study. The highest cloud point of F4 may be explained by the fact that the solubility of surfactant generally decreases with increasing temperature and starts to lose surface active properties. However, the presence of hydrophilic molecule such as PG in the aqueous phase can increase the solubility of the head group of surfactant in the aqueous phase (Lawrence and Rees, 2000). Thus, the solubility of surfactant is remained and the microemulsion is stabilized.

In vitro lipolysis is mimicking the intestinal lipid digestion process as suggested by Benito-Gallo et. al (2015). Therefore, it was selected as a tool to predict the *in vivo* behavior of lipid based formulation, i.e. microemulsion in this study. In humans, pancreatic lipase plays an important role in the lipid digestion in small intestine. From this study, it was observed that, without pancreatic lipase, the microemulsion was floating on the top of the digestive buffer (Figure 1, left). Upon addition of pancreatic lipase, this enzyme incorporates into an oil/water interface where, in the presence of bile salt micelles and colipase, the enzyme activation occurs. The lipolysis process is then initiated. By determining the content of MSE in the aqueous phase, F4 was found to greatly enhance MSE digestion compared to the PG solution. The extent of digestion was confirmed by the amount of sodium hydroxide which was added during the lipolysis process. Sodium hydroxide is added into the system to neutralize free fatty acids which are by products of lipid digestion and to maintain the pH of the system. As can be seen in Figure 3, the amount of sodium hydroxide consumed during lipolysis of MSE-F4 was higher than that of MSE-PG solution, confirming the higher extent of digestion of MSE-F4.

The enhancing effect of F4 compared to the PG solution can be explained by the fact that the catalytic activity of pancreatic lipase is activated only at the oil/water interface. Upon activation, lipids are digested resulting in monoglycerides, fatty acids and lysophospholipids etc (Kalepu et al., 2013). These lipolytic products are arranged into micelles, unilamellar vesicles and multilamellar vesicles in the presence of bile salts and these structures enhance the solubilization and absorptive capacity of the small intestine. On the other hand, in the absence of lipid substrates, the lipase enzyme is not activated, resulting in low extent of digestion. In addition, the enhanced intestinal absorption by microemulsion may be due to surfactant-induced increment permeability which disrupts the structure of the lipid bilayer and opens the tight junction of the intestinal epithelium (Gundogdu et al., 2001).

Conclusion

In conclusion, this study demonstrates that the microemulsion formulation consisting of 10%caprylic/capric triglyceride/70% PEG-8 caprylic/capric glyceridesmixed with polyglyceryl-3 diisostearate (3:1)/10%water/10%propylene glycol can greatly enhance digestion of MSE in *in vitro* lipolysis model. Thus, it has potential to enhance oral bioavailability of MSE in *in vivo*. However, further study in animal trials is necessary to be confirmed.

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