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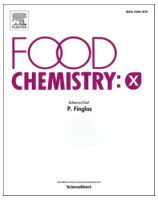

Self-constructed water-in-oil Pickering emulsions as a tool for increasing bioaccessibility of betulin

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ABSTRACT

Self-constructed water-in-oil emulsions can be stabilized by a natural pentacyclic triterpenoid, betulin. A higher betulin concentration (3%) results in smaller emulsion droplet sizes. Microscopy, confocal laser scanning microscopy and rheology indicate that the stabilizing mechanism is attributed to betulin crystals on the emulsion interface and within the continuous phase, thereby enabling excellent freeze/thaw and thermal stability. The betulin Pickering emulsion (1%) significantly increased betulin bioaccessibility (22.4%) compared to betulin alone (0.2%) and betulin-oil physical mixture (7.9%). A higher level of betulin at 3% leads to smaller emulsion particle size, potentially resulting in a greater surface area. This, in return, promotes a higher release of free fatty acids (FFA), contributing to the release and solubilization of betulin from emulsions. Additionally, it leads to the formation of micelles, further increasing betulin bioaccessibility (29.3%). This study demonstrates Pickering emulsions solely stabilized by phytochemical betulin provides an innovative way to improve its bioaccessibility.

1. Introduction

Betulin is a lupane-type triterpenoid characterized by an isopropylidene group and a five-membered ring. It possesses various health and medicinal effects, such as anti-inflammatory, anti-cancer, and antimicrobial properties (Amiri et al., 2020). Oral administration is the most common and convenient method for delivering betulin, making its oral efficiency crucial for realizing its functionalities. However, the low bioavailability and poor aqueous solubility severely limit the use of betulin as a potential therapeutic ingredient (Grymel, Zawojak, & Adamek, 2019).

Various systems have been developed to improve the solubility and delivery efficiency of betulin. *Lonicera japonica* leaf extract has been used as a stabilizer for betulin, achieving nearly 100% encapsulation efficiency (Yadav, Kumar, Kumari, & Yadav, 2016). This system exhibits excellent biocompatibility, high stability, and enhanced release characteristics (Yadav, Kumar, Kumari, & Yadav, 2016). Nanoparticles formed by zein or glycosylated zein and betulin using the antisolvent method have shown increased dispersion in water with 2.27 or 2.91-fold respectively compared to free betulin acid, resulting in enhanced solubility, sustained release, and inhibition of HepG2 cell proliferation (Peng, Jin, Wang, Wang, Xiao, & Xu, 2022). Organogelled emulsions failed to enhance the bioaccessibility of betulin but significantly increased its cell permeability, attributed to the high partition coefficient of betulin in oil, which promotes the formation of betulin mixed micelles during *in vitro* digestion (Ojeda-Serna et al., 2019).

The water-in-oil (W/O) emulsion is a widely used lipid-based food form that is considered a potential delivery system for water-soluble nutrients (Zembyla, Murray, & Sarkar, 2020). Additionally, Pickering emulsions are stabilized by insoluble solid particles that irreversibly adsorb onto the interface, forming a stable and steric barrier. Proteins and polysaccharides solid particles were commonly used for forming Pickering emulsions. For example, soy protein isolate and basil seed gum (Ocimum bacilicum L.) complex were used as solid particles under different pH for stabilizing Pickering emulsions (Naji-Tabasi, Mahdian, Arianfar, & Naji-Tabasi, 2021). The Pickering emulsion system prevents the aggregation of dispersed phases and improves the stability and delivery of bioactives (Mwangi, Lim, Low, Tey, & Chan, 2020; Xia, Xue, & Wei, 2021). Water-insoluble polyphenol crystals, such as polyphenol quercetin and curcumin, can be used as natural Pickering emulsion stabilizers to stabilize water droplets by the Pickering mechanism (Zembyla, Murray, & Sarkar, 2018). In comparison to curcumin particles, quercetin particles with a rod shape anchor more strongly at the interface (Zembyla et al., 2018). In our previous work, we investigated the ability of ursolic acid and diosgenin acting as Pickering stabilizers for stabilizing W/O emulsion, providing new strategy for constructing

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carrier free system for the utilization of these components (Liu, Xia, Guo, Lu, & Zeng, 2022; Wan, Xia, Guo, & Zeng, 2021). The greater significance of natural ingredients as W/O emulsion Pickering particles was that they were self-constructed stable delivery carriers, which was crucial for reassessing their performance in the process of digestion and absorption. Therefore, it was necessary to investigate whether betulin, the lupane-type triterpenoid have the ability to form W/O emulsion and its bioaccessibility can be promoted after it embedded in this self-constructed stable delivery system.

The present study aims to investigate the potential of betulin particles in stabilizing W/O Pickering emulsions for the first time. Additionally, it assesses the delivering efficiency of betulin through a special self-constructed emulsion using an *in vitro* gastrointestinal digestion model. The findings of this study not only expand the range of natural functional Pickering particles utilized to stabilize W/O emulsions, but also offer fresh insights into enhancing the effective delivery of hydrophobic bioactive ingredients.

2. Materials and methods

2.1. Materials

Betulin (≥98% purity) was purchased from Shanxi Jinkangtai Biological Technology Co., Ltd (Shanxi, China). Commercial rapeseed oil was obtained from a local supermarket. Nile red was obtained from Shanghai Ruiyong Biotechnology Co. Ltd. (China). Pepsin (≥250 U/mg), pancreatin (P7545, 8 × USP), and lipase (L3126, from porcine pancreas type II, 100–500 units/mg protein) were purchased from Sigma Chemical Company. Methanol was purchased from Hubei Fudun Science Technology Co., Ltd (Wuhan, China), and phosphoric acid was obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All other chemicals used were of analytical grade.

2.2. Scanning electron microscopy

The particle morphology of betulin was examined using a Czech TESCAN-MIRA3 high-resolution field emission scanning electron microscope (Geng, Jiang, Ma, Pu, Liu, & Liang, 2021). Betulin particles were uniformly distributed on the specimen stub using double-sided adhesive tape and sprayed with platinum.

2.3. Contact angle and surface/interfacial tension

Three-phase contact angles ($\theta_{\text{Betulin-water-oil}}$) were measured using a drop shape analysis system (DSA 100, KRUSS, Germany) following a previously reported method (Chen et al., 2018). The betulin particle was compressed into a thin film using a tablet machine at a pressure of 30 MPa. The film was placed into a liquid pool containing rapeseed oil for $\theta_{\text{Betulin-water-oil}}$ determination. Then, a water drop was extruded from a high-precision syringe and released on the film surface to become absorbed. The drop image was automatically recorded, and the corresponding contact angle was calculated using an ellipse-fitting model. Surface and interfacial tension were determined using the hanging drop method (Liu et al., 2022). The sample was loaded into a syringe, and a droplet was formed at the tip of the stainless steel needle surrounded by the oil phase, then the surface and interfacial tension was measured.

2.4. Preparation of emulsion

A betulin oil dispersion was prepared by dispersing it in rapeseed oil at a concentration of 0.5–3 wt%. To achieve a uniform betulin solution, the sample was placed in a constant temperature bath (HWCL-3, Zhengzhou Great Wall Technology & Trading Co., Ltd.) set at 80 °C and stirred at 400 rpm for 20 min. After cooling, a water-in-oil (W/O) emulsion was prepared by adding deionized water with varying water volume fractions (φ = 0.2–0.7) and stirring for 2 min at 12,000 rpm using an Ultra-Turrax T18 digital high-speed homogenizer. The resulting emulsion was immediately sealed in a 25 mL cylindrical tube with an inner diameter of 17 mm and stored at room temperature for further evaluation.

2.5. Microstructure and particle size

An appropriate amount of the emulsion was placed on a slide and covered for observation. The morphology was observed using a CX31 optical microscope (Beijing, China) equipped with a 100 × oil lens. As described in our previous report (Li, Li, Chen, Wang, Xie, & Sun, 2018), the oil phase was pre-stained with Nile red (excitation: 488 nm, emission: 610–630 nm, 0.1% in propylene glycol solution) before the preparation of the emulsion. One drop of the emulsion was placed onto a slide and covered with a cover slip. The droplet was observed using a Carl Zeiss LSM780 confocal laser scanning microscope (Oberkochen, Germany) equipped with an argon krypton laser. The obtained images were processed using ZEN 3.0 software (Carl Zeiss, Oberkochen, Germany). The particle size distribution and appearance of the dispersed droplets were recorded using a polarized light microscope (PLM) and analyzed using microstructure image analysis software (Nano measurer 1.2, Fudan University, China). The determination of particle size was achieved by capturing multiple micrographs using a 100x oil immersion lens. In order to obtain a statistically reasonable distribution of droplet size distribution, at least 200 droplets were analyzed for each emulsion sample. The Santer average diameter ($d_{3,2}$) was obtained by Eq. (1) (n-number of droplets).

$$d_{3,2} = \frac{\Sigma_{i=1}^{n} d_{P,i}^{3}}{\Sigma_{i=1}^{n} d_{P,i}^{2}} \tag{1}$$

2.6. Rheological charateristics

The rheological behavior of the W/O emulsion was determined using a Kinexus Rotary Rheometer (Malvern, UK). A parallel plate geometry with a diameter of 40 mm and the gap of 1 mm was utilized. Within the linear viscoelastic region (LVR) at 25 °C, oscillatory experiments including amplitude sweeps with stress ranging from 0.1 to 1000 Pa at the frequency of 1 Hz and frequency sweeps ranging from 0.1 to 100 rad s^{-1} at the stress of 1 Pa were conducted. Additionally, flow measurements were carried out by applying different shear rates (0.1–50 s^{-1}). The thixotropic behavior of the emulsion was investigated by evaluating the viscosity of emulsion gels over time under different shear rates. The shear methods of three interval thixotropy analyses were 0.1 s^{-1} for 180 s, 10 s^{-1} for 180 s, and 0.1 s^{-1} for 180 s with a constant frequency of 1 Hz, and a strain of 0.01%.

2.7. Stability of emulsion

The freeze/thawing (F/T) stability, centrifugation stability, and thermal stability of the emulsion were measured based on published references (Douaire et al., 2014; Ghosh & Rousseau, 2009; Wan et al., 2021). For the F/T stability test, samples were frozen in a − 17 °C freezer for 20 h and then thawed at 25 °C for 1 h. The stability of the emulsion after different F/T cycles was assessed, and the appearance was observed using a digital camera (Samsung nx mini, 9 mm). For centrifugation stability, the formatted emulsion samples (7.65 g) were transferred into centrifuge tubes (10 mL) were centrifuged at 5000 rpm for 5 min. The appearance was observed, and the oiling off was analyzed by calculating the ratio of the weight of the oil phase to the weight of the emulsion. For thermal stability, the samples were subjected to thermal stress in a constant temperature water bath at different temperature (30 °C, 50 °C, 80 °C) for 15 min. The stability was characterized by visual observation.

2.8. In vitro digestion

An *in vitro* digestion model comprising oral, gastric, and intestinal phases was used to evaluate the lipid hydrolysis of the emulsions, following a publication with some modifications (Mao & McClements, 2012). For the oral phase, betulin particles (5 mg, 10 mg, 20 mg), 0.5 g of oil, 0.5 g oil plus betulin (1%) physical mix (Betulin + oil) or 0.71 g Pickering emulsion (φ = 0.3, 1%, 2% and 3% betulin levels) were mixed with simulated saliva fluid (10 mL, pH 6.8) and incubated at 37 °C for 10 min. For the gastric phase, samples that originated from the oral phase were mixed with the simulated gastric fluid (20 mL). The pH of mixtures was adjusted to 2 and incubated at 37 °C for 2 h. For the intestinal phase, salt solution (1.5 mL) consisting of $CaCl_2$ (36.7 mg/mL) and NaCl (218.7 mg/mL) and bile extract solution (3.5 mL, pH 7.0) with concentration of 53.6 mg/mL in PBS were added. The pH was adjusted to 7.0, and enzyme suspension (2.5 mL, containing 60 mg of pancreatin and 60 mg of lipase in PBS, pH 7.0) were added. The mixture was maintained at 37 °C with continuous stirring (100 rpm) for 2 h. The free fatty acids (FFA) were determined using a pH-stat method and calculated using Eq. (2):

$$\%FFA = \frac{V_{NaOH}\ C_{NaOH}\ M_{lipid}}{2W_{lipid}} \times 100 \quad (2)$$

where V_{NaOH} and C_{NaOH} stand for the volume and concentration of NaOH, respectively; W_{lipid} was the total mass of canola oil, and M_{lipid} represented molecular mass of canola oil (931.6 g/mol).

The bioaccessibility of betulin was verified following the reported method (Yi, Li, Zhong, & Yokoyama, 2014). Samples obtained from the intestinal phase were centrifuged at 10,000 rpm (Thermo-Fisher ultracentrifuge) for 5 min at 4 °C. Then the micellar fraction was recovered and extracted with ethanol for 2 h at room temperature. Subsequently, it was filtered for HPLC analysis using a 0.22 μm pore size filter. The HPLC conditions included an injection volume of 20 μL, a YMC-Pack ODS-A column (250 mm × 4.60 mm) with a column temperature of 30 °C, and a mobile phase consisting of methanol/0.1% phosphoric acid (92.5:7.5, v/v). The elution time was 10 min with a flow rate of 1 mL/min, and the DAD detector was set at 210 nm.

The biaccessibility (%) was calculated by Eq. (3):

$$\text{Bioaccessibility}\ (\%) = \frac{\text{Amount of betulin in the final micelllar(mg)}}{\text{Initial betulin amount (mg)}} \times 100 \quad (3)$$

2.9. Statistical analysis

The results were calculated from three replicates and expressed as mean ± standard deviation. One-way ANOVA and Duncan's multiple range tests were conducted using SPSS version 27.0 (IBM, Armonk, NY, USA) to identify differences within each treatment group. The criterion for statistical significance was set at $P < 0.05$.

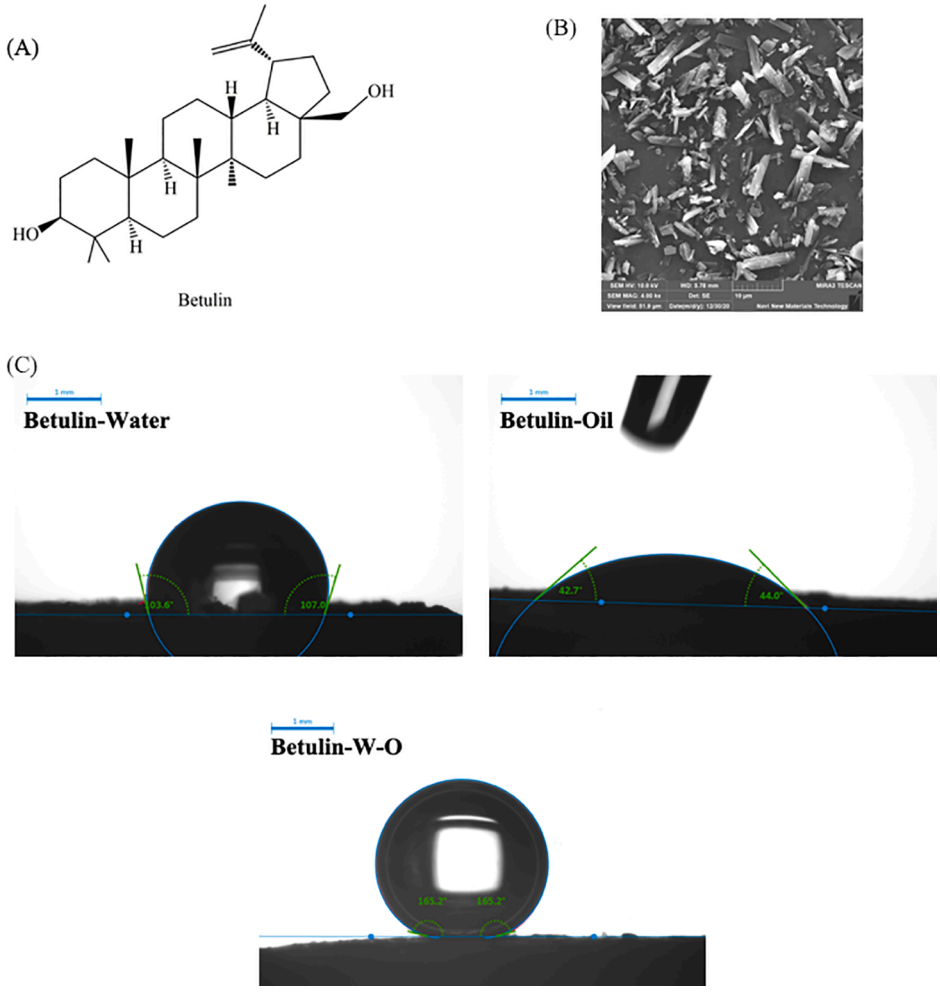


Fig. 1. (A) Molecular structure of betulin; (B) Scanning electron micrograph of betulin; (C) Two-phase/three-phase contact angle of betulin particles.

3. Results and discussion

3.1. Characteristics of betulin particles and betulin emulsions

The characteristics of betulin were shown in Fig. 1A&B. The particles shape played a pivotal role in emulsion formation. As shown in Fig. 1B, betulin crystals appeared as regular shaped rods within a size range of 1 to 10 μm, consistent with previous research (Şoica et al., 2012). In addition to particle morphology, wettability emerging as a critical parameter influencing emulsify properties. This property can be evaluated by gauging the tendency of a liquid to spread on a solid surface, denoted by the contact angle θ (Wang, Wu, Cao, Li, & Liao, 2020). Typically, a moderately hydrophobic particle, characterized by a water contact angle<90°, tends to facilitate O/W emulsion formation, whereas a hydrophobic particle with a contact angle>90° tends to favor W/O emulsion stabilization (Binks & Lumsdon, 2000). The contact angle of betulin particles on water was 105.36°, while the contact angle of betulin on rapeseed oil was 43.88° (Fig. 1C & Table 1). These results indicate strong hydrophobicity of betulin, suggesting its potential for W/O emulsion formation. The three-phase contact angle ($\theta_{betulin\text{-}water\text{-}oil}$), which refers to the angle formed by betulin particles at the interface, was 163.83°, closely resembling the value obtained when employing triterpene ursolic acid as the stabilizing agent (Liu et al., 2022).

The interfacial stabilization of emulsions relies on the particle cohesion and the interaction among atoms, ions, and molecules (Geng, Jiang, Ma, Pu, Liu, & Liang, 2021). Generally, emulsifiers such as surfactants have the ability to reduce interfacial tension, thereby promoting droplet dispersion and provide kinetic stability to emulsions. However, it is noteworthy that the introduction of betulin particles led to a significant increase in interfacial tensions. The oil-air surface tension value rose from 31.76 ± 0.01 to 35.05 ± 0.04 mN/m and the oil–water interfacial tension value increased from 32.58 ± 0.02 to 48.70 ± 0.04 mN/m (Table 1). It is important to emphasize that the reduction in interfacial tension does not always result in enhanced emulsion stability (Gaonkar, 1991). Furthermore, interfacial tension measurements solely quantify the adsorption of particles, while stability is influenced by the structure and orientation of stabilizer molecules at the interface (Gaonkar, 1991). Direct evidence indicates that the decrease in interfacial tension is not the primary stabilizing mechanism in Pickering emulsions. Instead, Pickering emulsions are characterized by higher surface loading and thickness (Xia, Xue, & Wei, 2021). Consequently, these results indicate that the formation of Pickering emulsions is driven by steric hindrance and surface rheology effects rather than the reduction of interfacial tension.

Next, the effects of betulin concentration and the water-to-oil ratio on the formation of emulsions were investigated. As illustrated in Fig. 2A, with the aqueous phase φ constant at 0.2, different concentrations of betulin particles (0.5–3%) were used to obtain the emulsions. A betulin concentration of 0.5% formed a naturally flowing emulsion, whereas stable self-standing gel-like structures were successfully formed within the range of 1% to 3% betulin concentrastions (Fig. 2A). These results indicate that emulsions stabilized by betulin particles exhibit distinct characteristics following high-temperature (80 °C) emulsification and subsequent cooling. At a constant betulin concentration of 2%, the effects of different aqueous phases (φ = 0.2 to φ = 0.7) on the formation of emulsions were investigated. It became evident that oil–water separation occurred when the oil fraction was low. In this scenario, the upper phase diaplayed porous emulsion gel structure and the lower phase appeared as transparent water. This observation suggests that when the oil concentration was relatively low, a significant amount of betulin particles may remain within the continuous phase after complete adsorption onto the surface of oil droplets, resulting in phase separation, given that water is denser than oil after cooling.

As we know, the size and morphology of the emulsions play pivotal role in determining their stability and performance in food and medicine (Fu et al. 2021). The influences of betulin particle concentration and aqueous fraction φ on the size and morphology of the emulsions were depicted in Fig. 2B. As the aqueous phase φ decreased from 0.6 to 0.2, it bacame evident that more stable emulsions were formed. This observation indicates that an adequate oil phase offers a larger surface area for betulin to effectively stabilize the emulsion. Furthermore, a reduction in the aqueous phase φ from 0.4 to 0.2 corresponded to the procduction of smaller droplets. When the oil phase remained constant, a higher concentration of betulin particles resulted in the formation of smaller emulsion droplets, as a surplus of particles was available to constitute the interface structure (Li, Liu, Xu, & Zhang, 2022). However, it seems contradictory that the size of betulin crystals, ranging from 1 to 10 μm, is similar to the size of the emulsions. This discrepancy may be caused by different betulin crystals formed under different systems, suggesting that betulin particles contribute to the interface and may have a smaller particle size (Spigno, Donsì, Amendola, Sessa, Ferrari, & De Faveri, 2013).

The microstructure of the emulsions stabilized by betulin particles was examined using CLSM, as shown in Fig. 2C. The emulsion droplets (black) of all samples exhibited even distribution as regular spheres, indicating that there was little or no coalescence during emulsion formation. As the betulin concentration increased from 1% to 3%, there was a noticeable rise in the quantity of betulin particles in the continuous phase. This effect was particularly pronounced at 3% betulin, where bright spots were observed both at the emulsion surface and continuous phase. These results suggest the coexistence of two possible modes for Pickering emulsion stabilization, one involving betulin particles adsorbed onto the droplets and the other involving particles forming bridges between the droplets. When the betulin concentration reached 3%, significantly smaller droplets were observed, consistent with the results obtained from particle size measurements.

Table 1
The two-phase and three-phase contact angle of betulin, water and rapeseed oil. Surface tension and interfacial tension with water of rapeseed oil and 0.1 wt% betulin oil suspensions.

	Contact angle (θ)		Surface tension & interfacial tension (mN/m)
Betulin-Water	105.36 ± 2.56[b]	Oil-Air	31.76 ± 0.01[d]
Betulin-Oil	43.88 ± 1.00[c]	$Oil_{betulin}$-Air	35.05 ± 0.04[b]
Betulin-W-O	163.83 ± 1.37[a]	Oil-Water	32.58 ± 0.02[c]
		$Oil_{betulin}$-Water	48.70 ± 0.04[a]

Data are Mean ± Standard deviation, different literals in same row indicate statistical difference ($p < 0.05$).

3.2. Rheological property of emulsions

The rheological behavior serves as an indicator of the intricate structure and characteristics of emulsion gels, which holds significant in their production, transportation, storage, and customer acceptance (Du, Li, Jiang, Tan, Liu, & Meng, 2021). As shown in Fig. 3A, the elastic modulus (G') was higher than the loss modulus (G") in the LVR, signifying that the emulsions possessed a predominantly elastic gel-like structure. This observation aligns with the previous visual appearance observed in Fig. 2. The G' values of the emulsions increased with rising betulin concentrations from 1% to 3%, indicating a potential enhancement in structural integrity due to the presence of betulin particles. With the betulin level fixed at 2%, the G' values decreased as the aqueous phase (φ) reduced from 0.4 to 0.2. Fig. 3B showed the relationship between frequency and rheological response. Emulsions are regarded as stable when G' > G" and G' and G" remain frequency independent, reflecting gel-like properties (Torres, Iturbe, Snowden, Chowdhry, & Leharne, 2007). The data revealed that the emulsion prepared using 1% betulin particles displayed an independent trend over frequency range

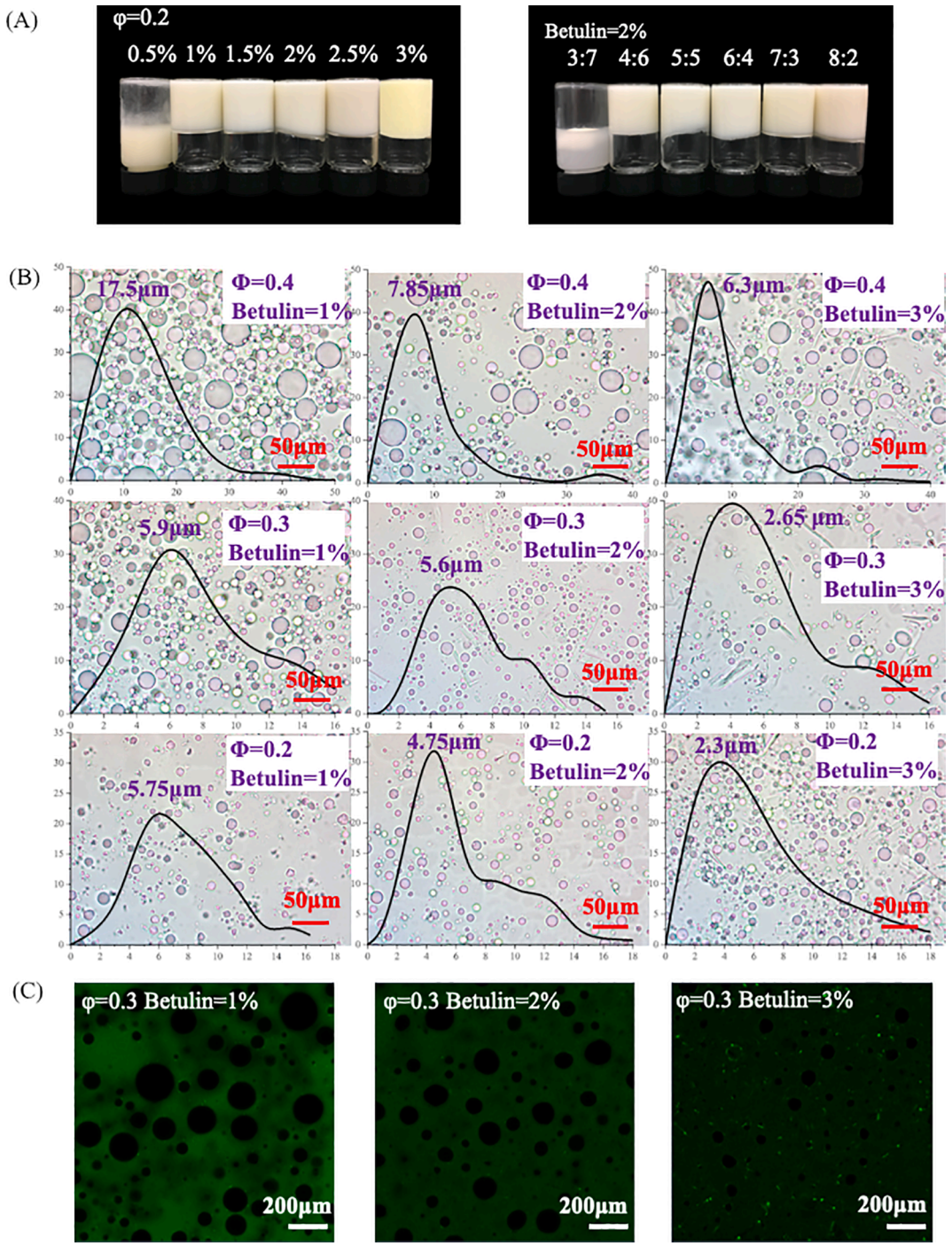


Fig. 2. Effects of betulin particle concentration and/or water fraction (φ) on (A) visual appearance (B) droplet size and (C) Confocal Laser Scanning Microscopy (CLSM) of the emulsions.

of 0.1 to 10 Hz. This indicates that the interface stabilized by betulin exhibited excellent elasticity against applied deformation and exhibited a predominant solid-like structure, as mentioned earlier. Furthermore, the other emulsions exhibited a more pronounced increase in response to higher frequencies. No yield point (G' = G") was found at any frequency, indicating that the emulsion gels did not undergo a gel-sol transition under higher deformation rates (Du et al., 2021). Collectively, these results provide further support that betulin particles are the primary contributors to the strength of the gel emulsions.

The apparent viscosity (η) in response to shear rate (0.1–50 s^{-1}) is presented in Fig. 3C, and all samples exhibit strong non-Newtonian pseudoplastic behavior, commonly known as shear thinning. Generally, the viscoelasticity of the emulsions is influenced by the factors such as droplet size distributions, inter-stabilizer interactions and rheology of the continuous phase (Du et al., 2021). As the betulin concentration increased, a higher apparent viscosity was observed, which can be attributed to the reinforcement network between the particle-stabilized oil droplets, consistent with the findings from CLSM results mentioned earlier. This phenomenon was positively correlated with our previous publication where ursolic acid was emplyed (Liu et al., 2022). It is worthnoting that an increased water droplets and hydrogen bonds results in an increase in hydrodynamic forces due to droplets interaction (Ariffin, Yahya, & Husin, 2016). Consequently, as the volume fraction of water increases, emulsions exhibit remarkable elasticity behavior

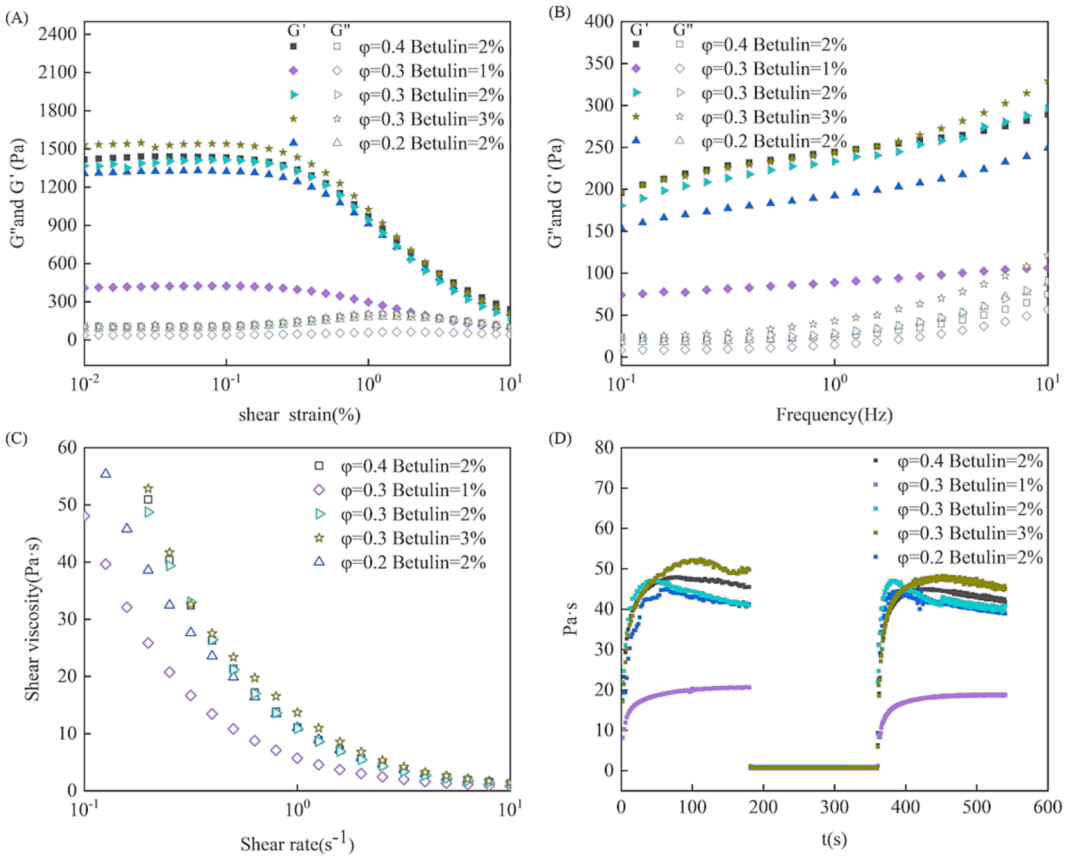


Fig. 3. Rheological properties of betulin particles stabilized W/O Pickering emulsions prepared with different betulin particle concentration and water fraction (φ) (A) Strain sweep curves (B) frequency sweep curves (C) apparent viscosity versus shear rate and (D) thixotropic recovery curves.

attributed by the interfacial energy related to the deformation of liquid films, resulting in elevated viscosity (Ariffin, Yahya, & Husin, 2016). However, when betulin is fixed at 2%, no significant distinction of shear viscosity was observed when varying internal water contents were applied. These results suggest that betulin exerts a more significant effect on the emulsion structure and the shear-thinning behavior of the emulsions due to the weak interaction between emulsion droplets, leading to a weak network structure among droplets (Xu et al., 2020).

A three-interval thixotropic test was conducted to investigate the structure-recovery property of Pickering emulsions stabilized by betulin (Fig. 3D). The samples underwent testing over three time periods, during which the viscosity of the sample was recorded over time under alternating cycles of different shear rates (0.1, 10, and 0.1 s^{-1}). In the first interval, no obvious trend observed in the variations of G' values for different aqueous phases. However, within the linear viscoelastic region, the G' values of the all emulsions significantly increased, especially when the betulin levels shifted from 1% to 3% at lower shear rates. This observation suggests that the presence of betulin altered the viscosity of the continuous phase and affected the movement of droplets. Nonetheless, the G' values of the emulsions experienced a sharp decrease when subjects to the shear force beyond the non-linear viscoelastic region, potentially indicating the instant destruction of the emulsion structure. In the third interval, as the shear rate was adjusted to the LVR, all emulsions exhibited robust thixotropic recovery with a percent recovery exceeding 70%, particularly for the betulin level at 1%. This finding indicates that the internal structures of the emulsions gradually recovered to their original state (Liu, Guo, Wan, Liu, Ruan, & Yang, 2018; Patel & Dewettinck, 2015).

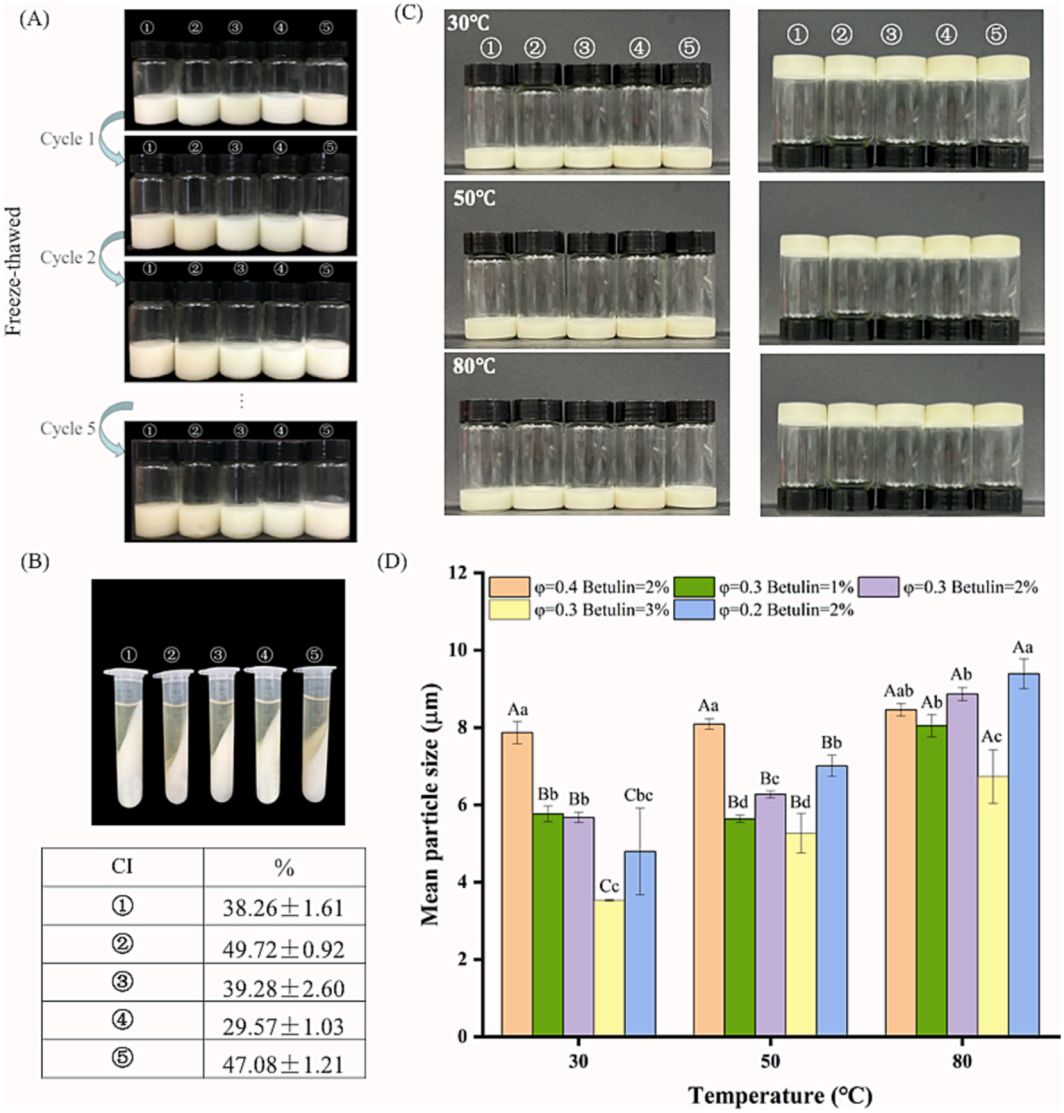
3.3. Stability of emulsions

3.3.1. Freeze/thaw stability

When emulsions undergo freeze, lipid droplets tend to aggregate within the freeze-concentrate phase, resulting in crystallization. Upon thawing, the lipid droplets gather together, causing instability phenomena including droplet coalescence, oil phase separation, and sedimentation, rendering them unsuitable for various food, chemicals, and medicine applications (Ghosh and Rousseau, 2009). Emulsions possessing a gel-like structure have the capability to prevent the formation of ice crystals (Wang, Singh, & Behrens, 2012). The Pickering stabilization mechanism provides superior freeze–thaw stability compared to conventional network-stabilized emulsions (Ghosh & Rousseau, 2011). The visual appearance of freshly prepared emulsions and emulsions subjected freeze–thaw treatment is shown in Fig. 4A. Betulin-stabilized Pickering emulsions exhibit remarkable stability even after five repeated freeze–thaw treatments, without experiencing any coalescence or creaming. This exceptional stability can be attributed to the inherent self-assembly of betulin into a robust interfacial structure, with the emulsion droplets effectively serving as space fillers within the emulsion gels (Li, Meng, Xu, & Zhang, 2021). Moreover, interactions may also form between the dispersed betulin particles and the continuous phase (Li et al., 2021).

3.3.2. Centrifugal stability

Centrifugation stability reflects the resistance of droplet coalescence and the long-term physical stability of emulsions. The occurrence of oiling off of all the emulsions was recorded after following centrifugation. A significant extent of oiling off, ranging from 29.6% to 50.0%, was observed among the emulsions. This phenomenon was likely due to insufficient quantity of betulin particles available to effectively adsorb



CI	%
①	38.26±1.61
②	49.72±0.92
③	39.28±2.60
④	29.57±1.03
⑤	47.08±1.21

Fig. 4. Visual appearances of the emulsions after (A) 1–3 times of Freeze/thawing treatment (B) centrifugation oiling off and (C) thermal treatment at 30, 50, and 80 °C; (D) mean particle size after thermal treatment. ①φ = 0.4 Betulin = 2% ②φ = 0.3 Betulin = 1% ③φ = 0.3 Betulin = 2% ④φ = 0.3 Betulin = 3% ⑤φ = 0.2 Betulin = 2%.

betulin molecules at the water droplets surface (Lan et al., 2022). As the betulin concentrations increased from 1% to 3%, there was notable reduction in oiling off, possibly attributed to the smaller particle size observed previously by higher concentration of betulin. It became apparent that larger emulsion droplets were more susceptible to coalescence (Langevin, 2019). At the same betulin level (2%), superior resistance to oiling off was achieved in the emulsion with a higher oil phase (φ = 0.2), and there was no significant difference between φ = 0.3 and φ = 0.4. These results suggest that an increased betulin content contributes to the centrifugal stability of the emulsions.

3.3.3. *Thermal stability*

Temperature plays a critical role in controlling the formation of interface crystals, consequently affecting the stability of emulsions (Zembyla et al., 2020). As shown in Fig. 4C, no discernible separation of the oil phase and water phase occurred when the emulsions were subjected to temperatures of 30, 50, and 80 °C for 15 min, demonstrating good thermal stability. The mean particle size of all emulsions ranged from 3.5 to 7.9 μm at 30 °C, 5.3 to 8.1 μm at 50 °C, and 6.7 to 9.4 μm at 80 °C (Fig. 4D). These variations can be attributed to droplet coalescence induced by high temperatures. A higher betulin level (3%) resulted in the lowest mean particle size, highlighting the importance of betulin crystals in enhancing the interfacial and network structures to stabilize Pickering emulsions. Emulsions stabilized by small molecular saponins exhibit deprived thermal stability due to the glycosidic linkages cleavage of saponins at elevated temperatures (Ralla et al., 2020). The thermal sensitivity of the stabilizing particles stands as a pivotal factor in Pickering emulsion stability, as their desorption energy typically exceeds the energy added through heating. Additionally, the temperature-responsive coalescence stability in W/O emulsions depends on the composition and spatial distribution of dispersed crystals, whether at the interface or within the continuous phase). The stabilization mechanism involving diosgenin crystals at the interface is attributed to the rigidity and resistance of diosgenin crystals to heat, a characteristic that bears some resemblance to betulin particles (Wan et al., 2021). Thus, all emulsions retained a sol-like consistency, with no visual changes following thermal treatment.

3.4. *Betulin bioaccessibility during in vitro gastrointestinal digestion*

In general, betulin particles exhibit low bioavailability (Grymel, Zawojak, & Adamek, 2019). Results in Fig. 5A showed that betulin alone

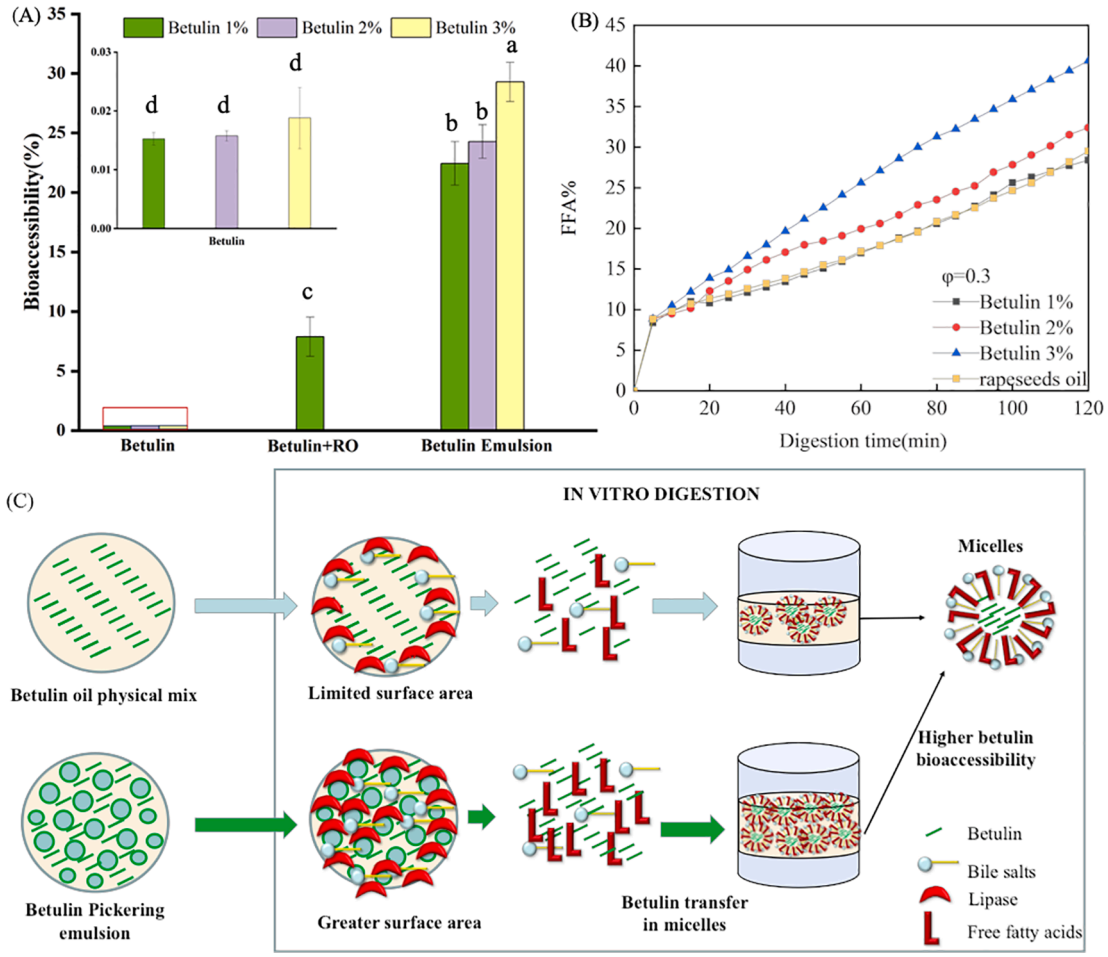


Fig. 5. (A) The bioaccessibility of betulin; (B) free fatty acids release of different Pikering emulsions; (C) The graphical mechanism illustration of betulin oil physical mix and Pickering emulsion *in vitro* digestion.

had very low bioaccessibility around 0.2%. Pickering emulsion-based systems have been developed for the encapsulation, protection, and facilitating the uptake of bioactives such as tocotrienols, carotenoids, and polyphenols (Mwangi, Lim, Low, Tey, & Chan, 2020). When betulin particles (1%) are emplyed as a Pickering stabilizer, their bioaccessibility was observed to be significantly higher (22.4%) than that of betulin physically mixed with oil (7.9%) following *in vitro* digestion. Emulsion with betulin concentration of 3% had the highest betulin bioaccessibility with 29.3%, which significantly higher than that concentration of 2% with bioaccessibility of 24.3%. Overall, the bioaccessibility of betulin increased with the increase of betulin concentration (1–3%) of all emulsions. This indicates that the emulsion structure resulted from higher betulin level may offer an increased contact surface for pancreatic lipase and bile salts, thereby enhancing the hydrolysis of oil into free fatty acids and accelerating the formation of betulin micelles. In the case of Pickering emulsions, higher concentrations of betulin demonstrated a pronounced effect on increasing the release of free fatty acids (FFA). Specifically, the final lipolysis rates at 120 min of digestion were as follows: 3% (40.6%) > 2% (32.4%) > 1% (28.4%) (Fig. 5B). This may be due to betulin particles leading to a greater surface area of water droplets exposed to lipase and bile salts in the small intestine, thereby promoting a higher rate and extent of lipid digestion (Winuprasith, Khomein, Mitbumrung, Suphantharika, Nitithamyong, & McClements, 2018).

The graphical illustration of the mechanism associated with the physical mixture of betulin oil and Pickering emulsion during digestion is shown in Fig. 5C. As the continuous phase is oil, it undergoes digestion, producing free fatty acids (FFA) when exposed to lipase and bile salts. Pickering emulsions have higher surface areas or interfaces compared to the physical mixture, resulting in a higher release of FFA. Since FFA is conducive to the formation of mixed micelles that can solubilize betulin, a higher level of betulin is transferred into micelles, resulting in increased bioaccessibility of betulin. Thus, natural betulin particles serve as the sole stabilizer for forming a super stable Pickering emulsion, and reciprocally, the Pickering emulsion enhances the bioaccessibility of betulin, which could contribute to the various health benefits of betulin.

4. Conclusion

In our study, we successfully produced novel self-constructed ultrastable W/O Pickering emulsions using only the natural bioactive compound betulin. Microscopy, CLSM, and rheological analysis results revealed the presence of two stabilizeing mechanisms, contingent on the simultaneous crystallization of betulin at the oil–water interface and its suspension in the continuous phase, effectively preventing droplet coalescence. All emulsions exhibited outstanding F/T and thermal stability. Higher concentrations of betulin resulted in the formation of smaller emulsion droplets, facilitating lipid digestion and enahncing the bioaccessibility of betulin. These findings underscore the potential for developing a range of emulsions exclusively stabilized by natural hydrophobic phytochemicals, thereby enhancing their bioaccessibility.

CRediT authorship contribution statement

Chaoxi Zeng: Conceptualization, Investigation, Formal analysis,

Methodology, Writing – original draft. **Yuxian Wang:** Data curation, Writing – original draft. **Yugang Liu:** Data curation, Writing – review & editing. **Shuxian Su:** . **Yuting Lu:** Data curation, Writing – review & editing. **Si Qin:** Conceptualization, Resources, Supervision, Writing – review & editing. **Meng Shi:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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