Andrographolide/ Phospholipid/ Cyclodextrin complex-loaded Nanoemulsion: Preparation, Optimization, in vitro and in vivo Evaluation

Linghui Zou, Wenya Ding, Qiuyan Huang, Xu Yang, Jilang Li, Tianyan Huang, Zeyu Li, Si Lin and Jianfang Feng

*Address for correspondence

Name: Jianfang Feng; Wenya Ding

E-mail: jfangfeng@126.com; DingWenYa666@163.com
Abstract

Andrographolide (AG), a natural product with various pharmacological effects, exhibited low oral bioavailability owing to its poor solubility, stability and low absorption. Previous studies have suggested that phospholipid (PC) and hydroxypropyl-β-cyclodextrin (HPCD) could improve the drug solubility and absorption. Moreover, nanoemulsion (NE) has been confirmed as an appropriate enhancer for oral bioavailability. Therefore, AG/HPCD/PC complex (AHPC) was synthesized, and AHPC-loaded nanoemulsion (AHPC-NE) was optimized and prepared using central composite design combined response surface methodology. The average droplet size and PDI were 116.50±5.99 nm and 0.29±0.03, respectively. AHPC-NE with a loading capacity of 0.32 ± 0.01% and an encapsulation efficiency of 96.43 ± 2.27% appeared round and uniformly dispersed based on transmission electron microscopy. In vivo release studies demonstrated that AHPC-NE had good sustained-release effects. Further, AHPC-NE significantly enhanced the absorption of AG with a relative bioavailability of 550.71% compared to AG suspension. Such findings reveal AHPC-NE as a potential strategy for sustained-release and oral bioavailability enhancement.

Keywords: andrographolide, nanoemulsion, optimization, hydroxypropyl-β-cyclodextrin, phospholipid, oral bioavailability.
INTRODUCTION

Andrographolide (AG), a diterpene lactone compound, is one of the main active constituents in the aerial parts and leaves of the plant *Andrographis paniculata*.\(^1\) Modern pharmacological studies revealed that AG has good performance in antibacterial,\(^2\) antivirus,\(^3\) antitumor,\(^4\) anti-inflammatory,\(^5\) and immune regulation.\(^6\) However, AG is considered as a class IV drug according to the biopharmaceutics classification system (BCS) with low solubility and low permeability.\(^7\) Despite the traditional preparations of AG used in clinical treatment such as dropping pills, tablets, and capsules have a certain therapeutic effect, the oral bioavailability remains at a low level, which is owing to their poor water solubility, rapid drug release and metabolism, and low intestinal absorption. Therefore, development of a new oral preparation of AG with high bioavailability has great potential for clinical application.

Recently, nanoemulsion (NE) has been reported as an excellent drug delivery system for drug efficiency enhancement.\(^9\) Specifically, the solubility can be greatly improved since the drug-loaded droplets could disperse in the continuous phase through the nanotechnology. Further, the encapsulation of the continuous phase can make the drug release slowly, which is beneficial to achieve long-acting effects due to the sustained-release properties can prevent the drug from being prematurely released and destroyed by gastric fluid or enzymes.\(^10\) Additionally, the favorable biocompatibility of phospholipid (PC) allows it to penetrate the cell membranes easily, and studies have reported that PC is capable of enhancing the solubility of drugs due to their amphiphilic nature, thereby improving their permeation and oral absorption\.\(^11,12\) For another, hydroxypropyl-\(\beta\)-cyclodextrin (HPCD) has already been confirmed as an excellent enhancer of drug solubility.\(^13\) Meanwhile, the carrier formed by combinations of different carriers may exhibit the advantages of each single carrier, which have a synergistic effect on drug delivery\(^14\). Therefore, the technology of NE combined with PC/HPCD complex for enhancing the oral bioavailability of AG is feasible and realistic.

Herein, AG/PC/HPCD complex (AHPC) was synthesized via magnetic stirring. In order to further enhance the efficiency of AG delivery, AHPC-loaded NE (AHPC-NE) was optimized and prepared using central composite design combined response surface
methodology (CCD-RSM). The physicochemical properties were characterized and the AG release from AHPC-NE in vitro was investigated. Finally, the pharmacokinetic studies in vivo were also explored to verify whether the oral bioavailability of AG improved when AHPC-NE was employed for drug delivery.

MATERIALS AND METHODS

Animals

SPF-grade Sprague Dawley (SD) rats (200±20 g) were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd. The animal study was approved by the Ethics Committee of the Experimental Animal Centre of Guangxi University of Chinese Medicine (Nanning, China) and in strict accordance with the recommendations in the Guidelines for the Care and Use of Animal Experiments of the Science Council of Guangxi University of Chinese Medicine.

Materials

AG (crude drugs, purity: ≥98%) were purchased from Maclean Biochemical Co., Ltd. (Shanghai, China). PC was obtained from Shenyang Tianfeng Biopharmaceutical Co., Ltd. HPCD was supplied by Zibo Qianhui Biological Technology Co., Ltd. Glycerin was purchased from Xilong Science Co., Ltd. Isopropyl myristate (IPM), Soybean oil, Isopropyl palmitate (IPP), Sorbitan monolaurate (Span-20), Sorbitan Oleate (Span-80), Diethylene glycol ether (Transcutol HP), Polyoxyethylene hydrogenated castor oil (RH-40), 1,2-Propanediol were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Glyceryl Monocaprylate (GMC) was obtained from Jiaozuo Minxin Industrial Co., Ltd. (Jiaozuo, China). Polyethylene glycol 400 (PEG-400) was supplied by Beijing Solarbio Technology Co., Ltd. (Beijing, China). All other chemicals and organic solvents were of analytical grade.

2.1 Preparation and Solubility of AHPC

2.1.1 Preparation of AHPC
AHPC was prepared by magnetic stirring method. Briefly, 85 mg of AG, 280 mg of PC, and 570 mg HPCD weighed individually were placed in a 50 mL round-bottomed flask, and dissolved by 20 mL of absolute ethanol. The mixture was magnetically stirred (DF-101S thermostatic magnetic stirrer, Bangxi Instrument Technology Co., Ltd., Shanghai, China) in a 40 °C water bath for 3.5 h. Finally, the absolute ethanol was evaporated under reduced pressure using a rotary evaporator (RV10 digital, IKA, Staufen, Germany) at 40 °C for 30 min (vacuum degree is 0.08) to obtain the AHPC.

2.1.2 Solubility Determination

An e2695 HPLC system (Waters, USA) with an Acclaim C18 column (4.6 mm × 250 mm × 5.0 µm) (Thermo, USA) was used for the determination of drug content. The chromatographic conditions were as follows, the mobile phase was consisted of 60:40 (v/v) methanol (eluent A) and water (eluent C), the volume of injection: 10 μL, the wavelength for the detection: 225 nm, flow rate: 1.0 mL/min, and temperature: 37°C. Excessive amounts of AHPC and AG were added into the solution with different pH (1.0, 3.0, 5.0, 6.8, 7.4) and the distilled water. 10 mL of the mixture was vortexed for 30 s and shaken at 25°C for 24 h to reach the equilibrium. The samples were centrifuged at 13000 rpm for 15 min, the supernatant was then collected and the solubility of AG was detected by the HPLC method above.

2.2 Process Optimization of AHPC-NE by CCD-RSM

2.2.1 Determination of Phase Solubility

Selection of oil phase, surfactant, and co-surfactant are critical parameters in the fabrication of nanoemulsion. Surfactants are amphiphilic molecules which stabilize nanoemulsion by reducing interfacial tension, and prevent droplet aggregation. Co-surfactants are used to complement surfactants, as they fit suitably in between structurally weaker areas, fortifying the interfacial film. And the addition of a second surfactant could provide more stable nanoemulsion with the minimum size than only one surfactant.\textsuperscript{15,16} To increase the AG loading capacity of AHPC-NE, these phases should dissolve as much AHPC as possible. Determination of phase solubility was carried out to select promising phase solvents.\textsuperscript{17}
According to the previous reported literatures, the alternative phase solvents were selected as follows: Oil phase: GMC, IPM, Soybean oil, IPP; Surfactant: Span-20, Span-80, RH-40; Co-surfactant: Transcutol HP, PEG-400, Glycerin, 1,2-Propanediol. Excessive amounts of AHPC were respectively added to 5 mL of the above phase solvents. The samples were vortexed for 5 minutes, sonicated for 20 minutes, and equilibrated for 24 hours at 37 ℃ using a constant temperature shaker. The saturated solution was taken for centrifugation at 8000 rpm for 10 min. Thereafter, 1 mL of supernatant was placed in a 5 mL volumetric flask, fixed with methanol, and filtered by the 0.45μm microporous membrane, the solubility of AG in different phase solvents was measured using the HPLC method described above.

2.2.2 Construction of Pseudo-ternary Phase Diagram

The mixed surfactant (S-mix) was composed of surfactant and co-surfactant, which were selected based on the results from the phase solubility experiment. Pseudo-ternary phase diagram was performed by water titration method combined emulsification method for the selection of Km (the mass ratio of surfactant and co-surfactant). The oil phase and S-mix with different Km (3:1, 2:1, 1:1, 1:2, 1:3) were mixed at a weight ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 respectively. The samples were homogenized by magnetic stirring for 5 min, the distilled water was then slowly added until the system became turbid. The amount of added distilled water was recorded, and the quality score of the three phase was calculated to construct the pseudo-ternary phase diagram.

2.2.3 Experimental Design

According to the results of the univariate experiment, the magnetic stirring speed (A), the amount of distilled water (B), and the amount of AHPC (C) were selected as the factors with value ranges of 1000-1800 rpm, 0.6-1.2 g, 50-450 mg, respectively. The average droplet size (Y1) and PDI (Y2) were selected as evaluation indicators in this experiment. In addition, overall desirability (OD) was set to reasonably reflect the influence of each factor according to the optimization requirements. The normalization method was used to fit each indicator, we
firstly standardized all indicators to a normalized value (desirability) between 0 and 1.0. The formula for calculation of desirability and OD are as follows:

\[
d_{\text{min}} = \frac{(Y_{\text{max}} - Y_i)}{(Y_{\text{max}} - Y_{\text{min}})}
\]  

\[
\text{OD} = (d_1 \times d_2 \times d_3 \times \ldots \times d_k)^{1/k}
\]

where \(d_{\text{min}}\) is the desirability of the indicator more favorable with a smaller value such as the droplet size and PDI, \(Y_i\) is the value of them in different experimental design, \(k\) is the number of evaluation indicators.

Thereafter, the horizontal code value and corresponding value of the three factors were listed in Table 1 and a total number of twenty runs was performed and their OD values were calculated in Table 2 based on the principle of CCD-RSM.\(^{25}\) The runs were operated in a randomized arrangement to avoid systematic bias.

**Table 1: Horizontal code value and corresponding value of the three factors**

| Factor | Horizontal code value |
|--------|-----------------------|
|        | -1.682    | -1      | 0      | 1      | 1.682  |
| A (rpm) | 1000      | 1162.2  | 1400   | 1637.8 | 1800   |
| B (g)   | 0.600     | 0.722   | 0.900  | 1.078  | 1.200  |
| C (mg)  | 50        | 131.09  | 250    | 368.91 | 450    |
Table 2: The measured response and OD values of central composite design

| NO. | stirring rate /rpm | m(water) /g | m(AHPC) /mg | Droplet size/nm | PDI Y₁ | PDI Y₂ | OD |
|-----|-------------------|------------|-------------|-----------------|--------|--------|-----|
| 1   | 1162.16           | 0.72       | 131.08      | 136.90          | 0.41   | 0.803  |
| 2   | 1637.84           | 0.72       | 131.08      | 212.10          | 0.28   | 0.890  |
| 3   | 1162.16           | 1.08       | 131.08      | 166.00          | 0.31   | 0.910  |
| 4   | 1637.84           | 1.08       | 131.08      | 158.10          | 0.30   | 0.928  |
| 5   | 1162.16           | 0.72       | 368.92      | 579.40          | 0.65   | 0.000  |
| 6   | 1637.84           | 0.72       | 368.92      | 518.70          | 0.58   | 0.170  |
| 7   | 1162.16           | 1.08       | 368.92      | 229.73          | 0.35   | 0.787  |
| 8   | 1637.84           | 1.08       | 368.92      | 166.30          | 0.38   | 0.816  |
| 9   | 1000.00           | 0.90       | 250.00      | 114.90          | 0.49   | 0.679  |
| 10  | 1800.00           | 0.90       | 250.00      | 204.20          | 0.28   | 0.894  |
| 11  | 1400.00           | 0.60       | 250.00      | 379.40          | 0.67   | 0.033  |
| 12  | 1400.00           | 1.20       | 250.00      | 169.00          | 0.30   | 0.913  |
| 13  | 1400.00           | 0.90       | 50.00       | 142.37          | 0.32   | 0.916  |
| 14  | 1400.00           | 0.90       | 450.00      | 374.90          | 0.43   | 0.523  |
| 15-20 | 1400.00       | 0.90       | 250.00      | 134.90          | 0.29   | 0.972  |

15-20 is repeatability runs, so the result is expressed by means

2.2.4 Optimization and Validation

The experimental design, analysis of variance (ANOVA), response surface analysis, and prediction of the optimal process were performed using the Design Expert (version 8.0.6.1). The quality of the fitting model was expressed by the P-value and $R^2$ (P-value < $0.1 \times 10^{-3}$ and a high $R^2$ value close to 1), which confirms the significance and accuracy of the applied model. Finally, AHPC-NE was prepared using the suggested optimal process to validate the prediction of model.

2.3 Characterization of the AHPC-NE

2.3.1 Droplet Size and PDI
3mL of AHPC-NE prepared by the optimal process was used to determine the droplet size, polydispersity index (PDI) with a Malvern Instrument (ZEN3700, Malvern Instruments Co., Ltd., Germany).

2.3.2 Transmission Electron Microscopy

To confirm the droplets size and investigate the morphological characteristics of AHPC-NE, transmission electron microscopy (TEM; HT-7700, Hitachi, Japan) was performed. 1mL of the NE was diluted by 3 times with GMC and then added dropwise on the copper mesh covered with carbon film. After natural drying, the microscopic appearance of the NE was observed using TEM.

2.3.3 Determination of LC and EE

The loading capacity (LC) and encapsulation efficiency (EE) were determined by following slight modifications of a previously reported indirect method\textsuperscript{26} and were calculated using the formulas given below. Briefly, 3mL of the NE and the NE without addition of AG were measured and weighed. The difference between the above two samples was the mass of AG, and the LC was calculated according to formula (3). On the other hand, 1 mL of AHPC-NE was placed in a 10 mL volumetric flask, 5 mL of methanol was added and sonicated for 30 minutes to demulsify the NE. The samples were fixed with an appropriate amount of methanol and then centrifuged in a low-temperature high-speed centrifuge (4 °C, 10000 r/min, 5 min). 1 mL of the supernatant was placed in a 10 mL volumetric flask, diluted with methanol to meet the volume and then measured by using the HPLC method mentioned above. The measured value indicates the amount of AG encapsulated in the NE, and EE was calculated according to formula (4).

\[
LC (\%) = \frac{\text{mass of AG}}{\text{mass of AHPC--NE formulations}} \times 100 \quad (3)
\]

\[
EE (\%) = \frac{\text{amount of AG encapsulated in NE}}{\text{amount of initial AG}} \times 100 \quad (4)
\]

2.4 Drug Release Studies
Drug release from AHPC-NE was assessed using the dialysis bag method. In brief, the dialysis bag (molecular retention capacity 8000-14000 Da) was boiled in water for 15 minutes, rinsed three times with pure water, shaken dry, and set aside. The dialysis bag (5 cm) was placed in the simulated gastric fluid (pH 1.2) and intestinal fluid (pH 6.8) for 12 h to reach the equilibrium of osmotic diffusion. After one end was clamped, 1 mL of AHPC-NE, AHPC solution, and AG suspension measured accurately were placed in a dialysis bag preliminarily filled with 1 mL release medium. The other end was quickly clamped and placed in a 250 mL beaker containing 100 mL release medium, The samples were shaken at a speed of 70 rpm and the temperature was controlled at 37±0.5°C. 1 mL of the release medium containing the diffused drug was collected after shaking for 0.5, 1, 2, 3, 5, 7, 9, 12, 24, 30, and 48 h. The same volume of release medium was replenished, and the sample solution was diluted 5-fold with the mobile phase, mixed well, and filtered using a microporous membrane (0.22um). The drug concentration was determined using the HPLC method and the cumulative drug release percentage (Q) was calculated using the following formula: (n=3)

$$Q(\%) = \frac{(C_i \times V_r) + (C_1 + C_2 + \ldots + C_{i-1}) \times V_s}{m} \times 100$$  (5)

where $C_i$ is the concentration of the samples collected at different time points, $V_r$ is the volume of the release medium (100 mL), $V_s$ is the volume of the sample (1mL), and $m$ is the mass of AG initially added.

### 2.5 Pharmacokinetic Studies

The plasma concentration of AG was determined using an HPLC-MS system (Ultimate Mate 3000/ TEQENDURA, Thermo Fisher Scientific, USA ) equipped with an ODS-C18 column (Hypersil GOLD, 2.1 mm × 100 mm × 1.9 μm, Thermo Fisher Scientific, USA). The mobile phases consisted of 0.1% formic acid (A) and (50:50, v/v) acetonitrile-methanol (B). The flow rate was 0.3 mL/min with a gradient elution of 20 to 95% B from 0 to 3 min, and then to 20% B from 5.5 to 5.6 min; the total run was 8 min and the column temperature was 40 °C. Bilobalide was selected as the internal standard. The following mass spectrometric parameters were used: TQD (triple quadrupole) detector with electrospray ionization (ESI) source; detection method, negative ion detection; ion source temperature, 350°C;
desolventizing temperature, 400°C; desolventizing gas flow, 800 L/h; scanning method, multiple ion reaction monitoring (MRM); scanning time, 0.6 s; quantitative ion, m/z 349.109→287.04 (AG) and m/z 325→162.968 (Bilobalide).

Twenty-four SD rats, which were fasted overnight with free access to water, were randomly divided into the following three groups: the first group was orally administered with AG suspension at a dosage of 30 mg/kg (AG was suspended in 2% (w/v) carboxymethyl cellulose aqueous solution); The second group was orally administered with AHPC solution at a dosage of 30 mg/kg (AHPC was dissolved in distilled water); The third group was orally administered with AHPC-NE at a dosage of 30 mg/kg. Subsequently, 300 μL of blood was collected from the ophthalmic veins of rats at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, and 48 h after oral administration. Blood samples were collected in polypropylene tubes containing heparin and centrifuged at 4000 rpm for 10 min. Thereafter, 1 mL of ethyl acetate was added to 100 μL of plasma containing 30 μL standard solution, the mixture was vortexed for 180 s to precipitate the proteins. The precipitated proteins were removed by centrifugation at 10000 rpm for 10 min, the supernatant was dried under nitrogen and the residue was dissolved by 200 μL of the mobile phase. The resulting solution was vortexed for 90 s and centrifuged at 13000 rpm for 10 min. An aliquot of 5 μL supernatant was injected into the HPLC-MS system mentioned above to determine the concentration of AG in plasma.

### 2.6 Data analysis

SPSS 25.0 was used for statistical analysis. Data obtained in triplicate at least three times repeated were expressed as mean ± SD. Differences between formulations were compared by one- or two-way ANOVA, $p < 0.05$ was considered significant.

### RESULTS

#### 3.1 Solubility of AHPC

As shown in Table 3, AHPC greatly increased the solubility of AG by 200-300 folds in solutions with different pH values, especially in the distilled water, the solubility of AG
reached the highest of 13.45±0.47 mg/mL when loaded in the PC/HPCD complex. There was a trend that the solubility of AHPC increased with increasing pH. However, the pure AG did not exhibit a significant change with the changing pH, and the highest solubility of 0.053±0.0009 mg/mL was still at a low level.

Table 3: Solubility of AHPC and pure AG in media at different pH values (mean ± SD, n=3)

| Medium        | Solubility (mg/mL) | AHPC       | Pure AG     |
|---------------|--------------------|------------|-------------|
| pH 1.0 HCl    | 9.81±0.18**        | 0.045±0.0019 |
| pH 3.0 HCl    | 10.68±0.11**       | 0.05±0.0005 |
| pH 5.0 ABS    | 11.35±0.13**       | 0.046±0.0017 |
| pH 6.8 PBS    | 11.37±0.13**       | 0.044±0.0014 |
| pH 7.4 PBS    | 11.93±0.06**       | 0.039±0.0006 |
| Distilled water | 13.45±0.47**     | 0.053±0.0009 |

HCl, hydrochloric acid; ABS, acetate buffer solution; PBS, phosphate buffer solution; AG, Andrographolide; AHPC, Andrographolide/phospholipid/Hydroxypropyl-β-cyclodextrin complex.**p < 0.05 compared with pure AG.

3.2 Phase Solubility of AHPC

The solubility results of AHPC in different oil phases can be seen from Fig 1A, GMC exhibited a better AHPC-solubilizing capacity with the saturated solubility of 268.33±40.97 μg/mL, which is much higher than IPP (29.55±2.27 μg/mL), IPM (5.66±0.90 μg/mL) and soybean oil (4.24±0.78 μg/mL). As to the results of surfactant showed in Fig. 1B, Span-20 demonstrated the highest solubility (37.31±2.58 μg/mL) followed by Span-80 (27.43±4.45 μg/mL) and RH-40 (13.85±0.77 μg/mL). In addition, this is clear from the results of co-surfactant in Fig. 1C that Transcutol HP with the saturated solubility up to 1872.39±201.85 μg/mL, indicating stronger solubilization effects than 1,2-Propanediol (1191.90±146.40 μg/mL), PEG-400 (1028.45±84.50 μg/mL), and Glycerin (467.37±94.58 μg/mL).
**Fig. 1:** Saturated solubility of AHPC in Oil phase (A), in Surfactant (B), and in Co-surfactant (C) (Mean ± SD; n=3). **p < 0.05 compared with other solution.

### 3.3 Pseudo-Ternary Phase Diagram

It can be clearly seen that the NE area (area under the curve) was affected by the Km (Fig.2), and the largest NE area was founded when the Km was 1:3. As depicted in Fig. 2E, the formed NE performed a clarified, transparent appearance with the maximum addition of distilled water when the mass ratio of GMC and S-mix was 2:3. Therefore, the final ratio of the ingredients was GMC (30.77%, w/w), S-mix (46.15%, w/w), and Distilled water up to 23.08% (w/w). In such conditions, the value range of the distilled water addition would increase in the univariate experiment, which is potentially helpful in optimizing the prescription with higher LC due to the greatly enhanced water solubility when AG prepared into AHPC. For the present, S-mix with a Km of 1:3 and the mass ratio of GMC and S-mix (2:3) were selected for further studies.
Fig. 2: Pseudo-ternary phase diagram of the NE prepared by water titration method under the conditions of $K_m=3:1$ (A), $K_m=2:1$ (B), $K_m=1:1$ (C), $K_m=1:2$ (D), $K_m=1:3$ (E). $K_m$, the mass ratio of surfactant and co-surfactant.

3.4 Fitting of Model and Response Analysis

The simultaneous effects of stirring speed (A), the amount of distilled water (B), and the amount of AHPC (C) on the OD acquired by CCD were generated into a response function mode: $OD=0.97+0.049A+0.23B–0.18C–0.026AB+0.012AC+0.16BC–0.06A^2–0.18B^2–0.083C^2$. ANOVA for RSM of the quadratic model demonstrated that the model is significant with a $P<0.0001$. All the linear terms A ($P=0.0406$), B and C ($P<0.0001$) have a significant effect. Moreover, $R^2$ and adjusted $R^2$ values of 0.9702 and 0.9435 are closed to 1, which indicated that the quadratic model was valid for the present study. The 3D surface plot in Fig. 3A showed the combined effect of distilled water addition and magnetic stirring speed on the OD value while the third factor was set to its middle value. It can be seen that the OD value decreased when both distilled water addition and magnetic stirring speed decreased. However,
if the amount of distilled water added got lower than 0.99 g, the OD value started to decrease rapidly. The interaction between magnetic stirring speed and AHPC addition when the other factor at its middle value was shown in Fig. 3B, the OD value increased inversely proportional to the amount of AHPC added. In addition, the interaction of distilled water addition and the AHPC addition was depicted in Fig. 3C, which indicated that the increase of distilled water addition and decrease of AHPC addition led to an increase of the OD value until the distilled water addition exceeded 0.99g; thereafter, the OD value started to decrease slightly. The results from Fig 3A and Fig 3C showed that too high or too low distilled water addition might cause a decrease in the OD value.
Fig. 3: 3D surface plots of the interaction between Amount of distilled water and Speed magnetic stirring (A), Speed of magnetic stirring and Amount of AHPC (B), Amount of distilled water and Amount of AHPC (C).

3.5 Method Optimization and Validation
In order to maximize the drug loading of the NE, we selected the prescription with the largest amount of AHPC added when the expected OD value was set as 1, the predicted optimization result associated with the maximum OD value of 1.0221 was reached when the experimental factors to their optimum values was set as follows: speed of magnetic stirring was 1453.33 rpm; amount of distilled water was 1.08 g, amount of AHPC was 223.33 mg. The OD value of AHPC-NE prepared via the optimized process is 1.013, which is in good concordance with the predicted OD value (deviation was 0.89%), supporting the accuracy of the optimization in the studied range using this model.

3.6 Characterization of AHPC-NE

As shown in Fig. 4, the droplet size distribution of the optimized AHPC-NE was generally uniform and the mean droplet size has already reached nano-level of 116.50±5.99 nm with a PDI of 0.29±0.03. The LC and EE of AHPC-NE were calculated to be 0.32± 0.01% and 96.43 ± 2.27%, respectively. The TEM image of the NE (Fig. 5) evidenced the nanostructures of AHPC-NE was a spherical shape, and the mean droplet size of 114.76 nm was calculated using a Nano-Measure software. The results showed good dispersity and uniformity with a size range of approximately 68.49 to 146.39 nm, which is in good agreement with that obtained from Malvern Instrument.

![Size Distribution by Intensity](image)

**Fig. 4:** Size distribution of the optimized AHPC-NE droplet determined by a Malvern Instrument.
Fig. 5: Transmission electron microscopy image and mean droplet size calculation of the AHPC-NE.

3.7 Drug Release Performance

Figure 6A shows the characteristics of AG release from AHPC-NE in simulated gastric fluid (pH=1.2). It can be seen that the drug in AG suspension group released almost completely at 12h, which was higher than that of AHPC solution and AHPC-NE. AHPC solution group reached the highest cumulative release rate of 65.98% at 9 h, while the AHPC-NE group reached the highest of 56.85% at 30 h. The drug release from the AHPC-NE showed an obvious sustained-release effect. On the other hand, it can be clearly seen from Fig. 6B that the cumulative drug release rate of each group has a significant increase when the pH increases to 6.8. The profile of AG suspension group reached the complete release at 7 h, the highest cumulative release rate of AHPC solution was 91.04% and 89.69% for AHPC-NE. Similar to drug release in the simulated gastric fluid (pH=1.2), the AG release from AHPC-NE also showed a characteristic of sustained-release.
3.8 Pharmacokinetics Studies

To verify whether AHPC-NE could further improve the oral bioavailability of AG in vivo, the pharmacokinetic evaluation of AHPC-NE, AHPC solution and AG suspension was performed in SD rats. The regress equation of A(AG)/A(Bilobalide, internal standard) (Y) to concentration of AG(X) was $Y=29.16X+45.23$ ($R=0.9998$) at range of 2-800 ng/mL. The plasma concentration-time profiles of AG after orally administered the three formulations was shown in Fig. 7 and the main pharmacokinetic parameters were listed in Table 4. The maximum plasma drug concentration ($C_{\text{max}}$) of AHPC-NE was 191.66±19.24 ng/mL, which was significantly different from that of AG suspension. With the AG suspension as a reference preparation, the relative bioavailability of the AHPC-NE and AHPC solution were 550.71% and 324.09%, respectively. In addition, the curves of AHPC-NE exhibited an excellent sustained-release effect that the $T_{\text{max}}$ and $t_{1/2}$ increased from 0.66±0.30 and 2.54±0.21 to 4.75±1.04 and 5.83±0.99 h when AHPC-NE was employed for drug delivery, which was also consistent with the results of the in vitro drug release experiment, the sustained-release of AG has a positive effect on prolonging the duration of drug action in vivo and then reducing the dosing frequency in clinical therapy.
Table 4: The main pharmacokinetic parameters of AHPC-NE, AHPC solution and AG suspension in SD rats (mean ± SD, n= 8).

| Parameters | Unit     | AHPC-NE          | AHPC solution    | AG suspension   |
|------------|----------|------------------|------------------|-----------------|
| C<sub>max</sub> | ng/mL    | 191.66±19.24**   | 256.54±58.92     | 66.85±5.57      |
| T<sub>max</sub> | h        | 4.75±1.04**      | 0.41±0.27        | 0.66±0.30       |
| AUC<sub>0-t</sub> | ng/mL·h  | 1225.71±26.31**  | 721.33±37.68     | 222.57±19.31    |
| MRT<sub>0-t</sub> | h        | 7.85±1.83**      | 2.21±0.71        | 3.07±0.75       |
| t<sub>1/2</sub> | h        | 5.83±0.99**      | 1.06±0.28        | 2.54±0.21       |
| Fr         | %        | 550.71%          | 324.09%          | -               |

C<sub>max</sub>, peak concentration; T<sub>max</sub>, time to peak concentration; t<sub>1/2</sub>, elimination half-life; AUC, area under curve; MRT, mean residence time in plasma; Fr, relative bioavailability with AG suspension; AG, Andrographolide; AHPC, Andrographolide/phospholipid/Hydroxypropyl-β-cyclodextrin complex; AHPC-NE, Andrographolide/phospholipid/Hydroxypropyl-β-cyclodextrin complex-loaded nanoemulsion. **p < 0.05 compared with AG suspension.

Fig. 7: Average plasma concentration-time curves of AG loaded in AHPC-NE, AHPC solution and AG suspension for oral drug delivery in SD rats. The dosage was 30 mg/kg (mean ± SD, n = 8).
DISCUSSION

The clinical applications of AG have been markedly limited owing to its poor solubility, stability, and low oral absorption. Therefore, it has a promising perspective to prepare a novel AG-loaded formulation with high oral bioavailability. To this aim, AHPC-NE drug delivery system was constructed to achieve efficient delivery for AG.

The synthesized AHPC significantly enhanced the solubility of AG in the solutions with different pH. Ren et al.\(^\text{30}\) and Singh et al.\(^\text{31}\) have prepared AG-CD complex and noted that the solubility of AG has a linear relationship with the concentration of CD, the lineal host-guest correlativity with slope (<1) advised synthesis of a 1:1 ratio complex of AG with CD. Zhang et al.\(^\text{32}\) have prepared AG-CD complex with an increased solubility of AG by approximately 31 folds. Unlike the binary complex, the AHPC consists of AG, PC, and HPCD with a molar ratio of 1:1.5:1.5 showed a 200-300 folds improvement at different pH ranges in the solubility test, which may be associated with the solubilization effect of HPCD and PC. Studies have reported that PC, an amphiphilic molecule with a hydrophilic nitrogen- or phosphorus-containing head and a long hydrophobic (lipophilic) hydrocarbon chain, could enhance the solubility of poorly soluble drugs.\(^\text{11,12}\) For another, the hydrophilic exterior surface and a non-polar interior cavity of HPCD would provide the suitably sized drug with a lipophilic microenvironment for hydrophobic drugs inclusion and finally improve their solubility.\(^\text{33}\) In addition, the solubility of AG in the AHPC group showed a trend of first increasing and then decreasing with the pH increasing, the possible reason was that HPCD at a strong acidic condition was unstable, causing the drug release from the interior cavity so that the inclusion solubilization effect was reduced at the acidic solution.\(^\text{34}\)

NE with a high LC is beneficial for clinical treatment due to the reduction of drug administration frequency. In order to enable more AHPC to be loaded into NE, we investigated the saturated solubility, an important criterion to choose each phase solvents, of AHPC in the alternative phase solvents mentioned previously. According to Fig. 1, GMC exhibited the highest solubilization of AHPC, the similar result was observed in a recent study by Elbardisy et al.\(^\text{35}\) Interestingly, the solubility of AHPC gradually decreases with the
The hydrophilic-lipophilic balance (HLB) value of the surfactant decreased, one possible explanation for this phenomenon was that higher HLB value indicated more hydrophilicity of the surfactant, and thus more AHPC can be dissolved due to their enhanced water solubility. However, Span-20 was too viscous as reported to achieve the expected emulsification effect in the actual production process while Span-80 displayed better. Therefore, GMC, Span-80, and Transcutol HP were selected as the oil phase, surfactant and co-surfactant, respectively. Thereafter, Pseudo-Ternary Phase Diagram was plotted to aid in selection of the Km and to determine the optimum mass ratio of GMC and S-mix.

In the analysis of response surface, we can find that the OD value was influenced greatly by the addition of distilled water. Such findings are in good agreement with the observations of Mayer et al. that the increase of the amount of distilled water added may change the droplet size and the PDI of NE. Meanwhile, the increase of AHPC addition would be helpful for the drug loading at a certain point. However, the OD value showed an obvious downward trend when the amount of AHPC added more than 250 mg. Therefore, the amount of AHPC addition should be carefully considered because its integrative effects on droplet size, PDI, and the LC.

The process of AHPC-NE was optimized using CCD-RSM and AHPC-NE with excellent physicochemical properties were successfully prepared and characterized. Compared with the AG-loaded NE prepared by Sooksai et al., AHPC-NE exhibited smaller droplet size and higher LC. Notably, the droplet size is critical to the efficacy of the NE. Win et al. found that the smaller the particle size, the faster the release of the standard polystyrene nanoparticles, which was related to the larger surface area per unit mass or volume. And the small droplet size could yield a favorable absorption for the application in vivo. Moreover, the higher LC may be helpful for reducing the dosing volume in the clinical applications.

In vitro drug release experiments were performed to preliminarily explore AG release properties from AHPC-NE, AHPC solution, and AG suspension in simulated gastric fluid and intestinal fluid. The absorption and metabolism of the drug in the gastrointestinal tract can be
estimated via the analysis of the drug release profiles. According to the drug release curve, we found that the AG loaded in AHPC released faster than that of AHPC-NE, which may be related to the encapsulation effect of oil phase of AHPC-NE slowed down the drug release rate. Another possible explanation for this phenomenon was that the encapsulation effect of the oil phase could also increase the time for fully mixed with the release medium. In addition, the cumulative drug release rate of each group increased with the pH increases to 6.8, this result was consistent with a study reported by Lee et al.\(^{42}\) This may be potentially relevant to the low stability of PC and HPCD in a strong acidic environment, the destruction of their structures might hinder the release of AG.\(^{43,44}\) Moreover, the result also indicated that the amount of AG released in the gastric fluid was much lower than that in the intestinal fluid, which is of great help to avoid AG being metabolized prematurely. Meanwhile, the cumulative release rate curves of AHPC solution as well as AHPC-NE showed a downward trend at the later time points, and this downward trend in the AHPC solution group was more pronounced, we speculated that this may be caused by the instability of AG in the release medium, since the continuous phase of AHPC-NE has a protective effect on AG\(^{45}\) and thus slowed down its degradation.

Pharmacokinetic studies revealed that AHPC-NE greatly improved the oral bioavailability of AG, which is consistent with those of Gao et al. and Yen et al.\(^{46,47}\) The possible reason was that the hydrophilic hydroxypropyl groups of HPCD increased the water solubility of AG, which was aided significantly in drug absorption, further, the amphiphilic PC molecule might stimulate the outer membrane of intestinal epithelial cells to guide the absorption of AG.\(^{48}\) In addition, the oil phase molecules in the prescription of AHPC-NE may penetrate into biological membranes and interact with phospholipid polar groups to change the fluidity of the membranes,\(^{49}\) and the formation of nano-sized droplets loaded with AHPC could further increase the transport of AG and intestinal absorption. Moreover, the nano-sized droplets were evenly dispersed in the continuous phase and this wrapping effect of the oil phase forms a natural barrier to reduce the metabolism of AG by gastric acid or other enzymes, thereby increasing the absorption of AG in the intestine,\(^{50}\) which was corresponds to the speculation in the drug release experiment. It is also worth noting that the \(C_{\text{max}}\) of
AHPC was higher than that of AHPC-NE while the $\text{AUC}_{0-t}$ was lower, which may be due to the sustained-release effect of AHPC-NE affecting the plasma concentration.\(^5\) Although the AHPC absorbed quickly to reach a higher $C_{\text{max}}$, the elimination rate was also fast, thereby causing a lower bioavailability.

In conclusion, we prepared the AHPC first and investigated its solubilization. The process of AHPC-NE was optimized using CCD-RSM to further increase the oral bioavailability. We assessed the characteristics and efficiency of the AHPC-NE in vitro and in vivo. Our results indicated that AHPC markedly enhanced AG solubility. In vitro release experiments exhibited a sustained-release of AG when loaded in the AHPC-NE, which is in agreement with our in vivo observations. In addition, pharmacokinetic studies showed that the optimized AHPC-NE significantly increased the oral bioavailability of AG with a relative bioavailability of 550.71% compared with the AG suspension. Such findings demonstrate that the AHPC-NE is effective for improving the oral bioavailability and sustained-release of AG, exhibiting great potential for clinical applications.

Acknowledgements:

This research was supported by the National Natural Science Foundation of China (No.82060806), Qihuang High-level Talent Team Cultivation Project of Guangxi University of Chinese Medicine (2021002) and Guangxi science and technology base and talent project (Guike AD20238058).

Conflict of interest

The authors declare no conflict of interest.

Ethics approval and consent to participate

The animal study was approved by the Ethics Committee of the Experimental Animal Centre of Guangxi University of Chinese Medicine (Nanning, China, No. SYXK-GUI-2019-0001).
References

1) Dai Y, Chen SR, Chai L, Zhao J, Wang Y, Wang Y. Overview of pharmacological activities of *Andrographis paniculata* and its major compound andrographolide. *Crit. Rev. Food. Sci. Nutr.*, **59**, S17-129 (2019).

2) Banerjee M, Parai D, Chattopadhyay S, Mukherjee SK. Andrographolide: antibacterial activity against common bacteria of human health concern and possible mechanism of action. *Folia Microbiol.*, **62**, 237-244 (2017).

3) Jiang M, Sheng F, Zhang Z, Ma X, Gao T, Fu C, Li P. *Andrographis paniculata* (Burm. f.) Nees and its major constituent andrographolide as potential antiviral agents. *J. Ethnopharmacol.*, **272**, 113954 (2021).

4) Hodroj MH, Jardaly A, Abi Raad S, Zouein A, Rizk S. Andrographolide potentiates the antitumor effect of topotecan in acute myeloid leukemia cells through an intrinsic apoptotic pathway. *Cancer Manage. Res.*, **10**, 1079 (2018).

5) Ye JF, Zhu H, Zhou Z-F, Xiong R-B, Wang X-W, Su L-X, Luo B-D. Protective mechanism of andrographolide against carbon tetrachloride-induced acute liver injury in mice. *Biol. Pharm. Bull.*, **34**, 1666-1670 (2011).

6) Zhang T, Zhu L, Li M, Hu Y, Zhang E, Jiang Q, Han G, Jin Y. Inhalable Andrographolide-β-cyclodextrin Inclusion Complexes for Treatment of Staphylococcus aureus Pneumonia by Regulating Immune Responses. *Mol. Pharmaceutics*, **14**, 1718-1725 (2017).

7) Roy P, Das S, Auddy RG, Saha A, Mukherjee A. Engineered andrographolide nanoparticles mitigate paracetamol hepatotoxicity in mice. *Pharm. Res.*, **30**, 1252-1262 (2013).

8) Gou J, Fei S, Xue B, Zhang J, Zhang Y, Wang X, Zhang Y, Yin T, He H, Tang X. Triacetylated andrographolide solid dispersions: Preparation, stability study and in vivo anti-inflammation in mice ulcerative colitis model. *J. Drug Deliv. Sci. Technol.*, **51**, 91-100 (2019).

9) Ikeuchi-Takahashi Y, Kobayashi A, Ishihara C, Matsubara T, Matsubara H, Onishi H. Influence of polysorbate 60 on formulation properties and bioavailability of morin-loaded nanoemulsions with and without low-saponification-degree polyvinyl alcohol. *Biol. Pharm. Bull.*, **41**, 754-760 (2018).
10) Jamali SN, Assadpour E, Jafari SM. Formulation and application of nanoemulsions for nutraceuticals and phytochemicals. *Curr. Med. Chem.*, 27, 3079-3095 (2020).

11) Khan J, Alexander A, Saraf S, Saraf S. Recent advances and future prospects of phyto-phospholipid complexation technique for improving pharmacokinetic profile of plant actives. *J. Controlled Release*, 168, 50-60 (2013).

12) Pu Y, Zhang X, Zhang Q, Wang B, Chen Y, Zang C, Wang Y, Dong TT-X, Zhang T. 20(S)-Protopanaxadiol phospholipid complex: process optimization, characterization, in vitro dissolution and molecular docking studies. *Molecules*, 21, 1396 (2016).

13) Li N, Wang N, Wu T, Qiu C, Wang X, Jiang S, Zhang Z, Liu T, Wei C, Wang T. Preparation of curcumin-hydroxypropyl-β-cyclodextrin inclusion complex by cosolvency-lyophilization procedure to enhance oral bioavailability of the drug. *Drug Dev. Ind. Pharm.*, 44, 1966-1974 (2018).

14) Cui W, Li J, Decher G. Self-assembled smart nanocarriers for targeted drug delivery. *Adv. Mater.*, 28, 1302-1311 (2016).

15) Singh Y, Meher JG, Raval K, Khan FA, Chaurasia M, Jain NK, Chourasia MK. Nanoemulsion: Concepts, development and applications in drug delivery. *J. Controlled Release*, 252, 28-49 (2017).

16) Peng L-C, Liu C-H, Kwan C-C, Huang K-F. Optimization of water-in-oil nanoemulsions by mixed surfactants. *Colloids Surf. A Physicochem. Eng. Asp.*, 370, 136-142 (2010).

17) Akhtar J, Siddiqui HH, Fareed S, Badruddeen, Khalid M, Aqil M. Nanoemulsion: for improved oral delivery of repaglinide. *Drug. Deliv.*, 23, 2026-2034 (2016).

18) Ali A, Ansari VA, Ahmad U, Akhtar J, Jahan A. Nanoemulsion: An advanced vehicle for efficient drug delivery. *Drug Res.*, 67, 617-631 (2017).

19) Yi T, Liu C, Zhang J, Wang F, Wang J, Zhang J. A new drug nanocrystal self-stabilized Pickering emulsion for oral delivery of silybin. *Eur. J. Pharm. Sci.*, 96, 420-427 (2017).

20) Seo YG, Kim DH, Ramasamy T, Kim JH, Marasini N, Oh Y-K, Kim D-W, Kim JK, Yong CS, Kim JO. Development of docetaxel-loaded solid self-nanoemulsifying drug delivery system (SNEDDS) for enhanced chemotherapeutic effect. *Int. J. Pharm.*, 452, 412-420 (2013).

21) Senapati PC, Sahoo SK, Sahu AN. Mixed surfactant based (SNEDDS) self-nanoemulsifying drug delivery system presenting efavirenz for enhancement of oral bioavailability. *Biomed. Pharmacother.*, 80, 42-51 (2016).
22) Barradas TN, de Campos VEB, Senna JP, Coutinho CdSC, Tebaldi BS, e Silva KGdH, Mansur CRE. Development and characterization of promising o/w nanoemulsions containing sweet fennel essential oil and non-ionic surfactants. *Colloids Surf., A*, 480, 214-221 (2015).

23) Jaiswal M, Dudhe R, Sharma PK. Nanoemulsion: an advanced mode of drug delivery system. *3 Biotech*, 5, 123-127 (2015).

24) Lacidogna G, Scaramozzino D, Carpinteri A. Optimization of diagrid geometry based on the desirability function approach. *Curved Layer. Struct.*, 7, 139-152 (2020).

25) Behera SK, Meena H, Chakraborty S, Meikap BC. Application of response surface methodology (RSM) for optimization of leaching parameters for ash reduction from low-grade coal. *Int. J. Min. Sci. Technol.*, 28, 621-629 (2018).

26) Ngampunwetchakul L, Toonkaew S, Supaphol P, Suwantong OJJoPR. Semi-solid poly (vinyl alcohol) hydrogels containing ginger essential oil encapsulated in chitosan nanoparticles for use in wound management. *J. Polym. Res.*, 26, 1-8 (2019).

27) Chen Y, Li G, Wu X, Chen Z, Hang J, Qin B, Chen S, Wang R. Self-microemulsifying drug delivery system (SMEDDS) of vinpocetine: formulation development and in vivo assessment. *Biol. Pharm. Bull.*, 31, 118-125 (2008).

28) Jaafar MHM, Hamid KA. Chitosan-coated alginate nanoparticles enhanced absorption profile of insulin via oral administration. *Curr. Drug Delivery*, 16, 672-686 (2019).

29) Laid TM, Abdelhamid K, Eddine LS, Abderrhmane B. Optimizing the biosynthesis parameters of iron oxide nanoparticles using central composite design. *J. Mol. Struct.*, 1229, 129497 (2021).

30) Ren K, Zhang Z, Li Y, Liu J, Zhao D, Zhao Y, Gong T. Physicochemical characteristics and oral bioavailability of andrographolide complexed with hydroxypropyl-β-cyclodextrin. *Pharmazie.*, 64, 515-520 (2009).

31) Singh SC, Khatri DK, Singh K, Kanchupalli VK, Madan J, Singh SB, Singh H. Molecular encapsulation of andrographolide in 2-hydroxypropyl-β-cyclodextrin cavity: synthesis, characterization, pharmacokinetic and in vitro antiviral activity analysis against SARS-CoV-2. *Heliyon*, 7, e07741 (2021).

32) Zhang D, Huang Y, Qiao Y, Xia C, Luo Y, Chen Z. Antibacterial activity of inclusion complexes of andrographolide and 14-acetylandrographolide by hydroxypropyl-β-cyclodextrin. *J. Nanjing Agricultural University*, 39, 318-324 (2016).
33) Loh GOK, Tan YTF, Peh KK. Enhancement of norfloxacin solubility via inclusion complexation with β-cyclodextrin and its derivative hydroxypropyl-β-cyclodextrin. *Asian J. Pharm.*, **11**, 536-546 (2016).

34) Gao Y, Li G, Zhou Z, Gao L, Tao Q. Sensitive complex micelles based on host-guest recognition from chitosan-graft-β-cyclodextrin for drug release. *Int. J. Biol. Macromol.*, **105**, 74-80 (2017).

35) Elbardisy B, Galal S, Abdelmonsif DA, Boraie N. Intranasal Tadalafil nanoemulsions: formulation, characterization and pharmacodynamic evaluation. *Pharm. Dev. Technol.*, **24**, 1083-1094 (2019).

36) Aziz ZAA, Nasir HM, Ahmad A, Setapar SHM, Ahmad H, Noor MHM, Rafatullah M, Khatoon A, Kausar MA, Ahmad I. Enrichment of Eucalyptus oil nanoemulsion by micellar nanotechnology: transdermal analgesic activity using hot plate test in rats' assay. *Sci. Rep.*, **9**, 13678-13678 (2019).

37) Matsumoto S, Sherman P. The viscosity of microemulsions. *J. Colloid Interface Sci.*, **30**, 525-536 (1969).

38) Mayer S, Weiss J, McClements DJ. Vitamin E-enriched nanoemulsions formed by emulsion phase inversion: factors influencing droplet size and stability. *J. Colloid Interface Sci.*, **402**, 122-130, (2013).

39) Sooksai N, Treesuppharat W, Theeramunkong S, Asasutjarit R. Andrographolide-loaded nanoemulsion and its activity against non-Melanoma skin cancer cells. *Trans. Tech. Publ.*, **819**, 139-144 (2019).

40) Win KY, Feng S-S. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. *Biomaterials*, **26**, 2713-2722 (2005).

41) Kawakami K, Yoshikawa T, Hayashi T, Nishihara Y, Masuda K. Microemulsion formulation for enhanced absorption of poorly soluble drugs: II. In vivo study. *J. Controlled. Release.*, **81**, 75-82 (2002).

42) Lee SH, Lee YS, Song JG, Han HK. Improved In vivo Effect of Chrysin as an Absorption Enhancer Via the Preparation of Ternary Solid Dispersion with Brij® L4 and Aminoclay. *Curr. Drug Delivery*, **16**, 86-92 (2019).

43) Manzanares MI, Solís V, de Rossi RH. Effect of cyclodextrins on the electrochemical behaviour of ascorbic acid on gold electrodes. *J. Electroanal. Chem.*, **407**, 141-147 (1996).
44) Zhong J, Wang Q, Qin X. Improving the stability of phosphatidylcholine-enhanced nanoemulsions using octenyl succinic anhydride-modified starch. *Int. J. Biol. Macromol.*, 120, 1500-1507 (2018).

45) Rachmawati H, Novel MA, Nisa RM, Berlian G, Tandrasasmita OM, Rahma A, Riani C, Tjandrawinata RR. Co-delivery of curcumin-loaded nanoemulsion and Phaleria macrocarpa extract to NIH 3T3 cell for antifibrosis. *J. Drug Delivery Sci. Technol.*, 39, 123-130 (2017).

46) Gao F, Zhang Z, Bu H, Huang Y, Gao Z, Shen J, Zhao C, Li Y. Nanoemulsion improves the oral absorption of candesartan cilexetil in rats: performance and mechanism. *J. Controlled Release*, 149, 168-174 (2011).

47) Yen CC, Chen YC, Wu MT, Wang CC, Wu YT. Nanoemulsion as a strategy for improving the oral bioavailability and anti-inflammatory activity of andrographolide. *Int. J. Nanomedicine*, 13, 669-680 (2018).

48) Shi C, Tong Q, Fang J, Wang C, Wu J, Wang W. Preparation, characterization and in vivo studies of amorphous solid dispersion of berberine with hydrogenated phosphatidylcholine. *Eur. J. Pharm. Sci.*, 74, 11-17 (2015).

49) Ganta S, Deshpande D, Korde A, Amiji M. A review of multifunctional nanoemulsion systems to overcome oral and CNS drug delivery barriers. *Mol. Membr. Biol.*, 27, 260-273 (2010).

50) Xu HY, Liu CS, Huang CL, Chen L, Zheng YR, Huang SH, Long XY. Nanoemulsion improves hypoglycemic efficacy of berberine by overcoming its gastrointestinal challenge. *Colloids Surf. B*, 181, 927-934 (2019).

51) Goodman AD, Brown TR, Cohen JA, Krupp LB, Schapiro R, Schwid SR, Cohen R, Marinucci LN, Blight AR. Dose comparison trial of sustained-release fampridine in multiple sclerosis. *Neurology*, 71, 1134-1141 (2008).