A new biocompatible microemulsion increases extraction yield and bioavailability of *Andrographis paniculata*

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[ABSTRACT] The purpose of this study was to design and prepare a biocompatible microemulsion of *Andrographis paniculata* (BMAP) containing both fat-soluble and water-soluble constituents. We determined the contents of active constituents of BMAP and evaluated its bioavailability. The biocompatible microemulsion (BM), containing lecithin and bile salts, was optimized in the present study, showing a good physical stability. The mean droplet size was 19.12 nm, and the average polydispersity index (PDI) was 0.153. The contents of andrographolide and dehydroandrographolide in BMAP, as determined by high performance liquid chromatography (HPLC), were higher than that in ethanol extraction. The pharmacokinetic results of BMAP showed that the $AUC_{0-7}$ and $AUC_{0\rightarrow\infty}$ values of BMAP were 2.267 and 27.156 μg·mL$^{-1}$·h$^{-1}$, respectively, and were about 1.41-fold and 6.30-fold greater than that of ethanol extraction, respectively. These results demonstrated that the bioavailability of andrographolide extracted by BMAP was significantly higher than that extracted by ethanol. In conclusion, the BMAP preparation displayed an improved dose form for future clinical applications.

[KEY WORDS] *Andrographis paniculata*; Biocompatible microemulsion; Andrographolide; Bioavailability; Ethanol extract

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Introduction

*Andrographis paniculata* (Burm.f.) nees, a famous Chinese medical plant, belongs to the family of Acanthaceae and contains a variety of bioactive ingredients, such as andrographolide, neo-andrographolide, deoxyandrographolide, and methoxy flavonoids [1]. According to the traditional Chinese medicine (TCM) theory, *Andrographis paniculata* (AP) can clear heat and toxicants, cool blood, and diminish swelling [2]. The major active constituents have positive effects on regulation of immunity [3] and anti-inflammatory [4], antibacterial, and antioxidant activities [5-6], making AP “natural antibiotics” and conferring high value in treatment of infectious [7-8], cardiovascular and cerebrovascular [9-10], digestive and other diseases [11-12]. In addition, these active constituents have also been studied for treatment of cancer [13], lung injury and mental illnesses [14-15].

Water, ethanol, and alkali extraction and acid precipitation are frequently used to extract active ingredients of AP for medicinal purposes at present, but these methods have certain drawbacks. Some components in AP can be extracted by water, but the extraction yield for andrographolide is poor. The yield of andrographolide is high with ethanol extraction, and ethanol in moderate concentration is also suitable to extract flavonoid glycosides, but has some defects like time-consuming, use of large volume of solvents and lower yield, resulting in waste of resources. The ring-opening derivatives of andrographolide are soluble in water with alkali extraction method, so a better extraction yield for andrographolide is poor. The yield of andrographolide is high with ethanol extraction, and ethanol in moderate concentration is also suitable to extract flavonoid glycosides, but has some defects like time-consuming, use of large volume of solvents and lower yield, resulting in waste of resources. The ring-opening derivatives of andrographolide are soluble in water with alkali extraction method, so a better extraction yield for andrographolide and dehydroandrographolide can be obtained, but the extraction rate of total flavonoids in an alkaline environment is significantly decreased at the same time [16-18]. Microwave-assisted extraction (MAE) [19], decompressing inner ebullition (DIE)

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Simultaneously solubilize the components with various polarities remains elusive. Therefore, finding proper solvents to simultaneously solubilize all of the active ingredients with different polarities in AP conditions. Some improved traditional extraction technologies used in recent years, because of expensive or stringent conditions. Some improved traditional extraction technologies can also improve the extraction yield of traditional Chinese medicines [25-26], but whether these methods could solubilize all of the active ingredients with different polarities in AP remains elusive. Therefore, finding proper solvents to simultaneously solubilize the components with various polarities is the key to extract active ingredients of AP comprehensively and effectively.

Microemulsion is a transparent or translucent isotropic colloidal system that spontaneously forms from the combination of water, oil, surfactant and co-surfactant in a proper proportion. As a thermodynamically stable system, it can be used as pharmaceutical carrier to solubilize different polarity drugs [27-28], which may be suitable for extracting all the active ingredients of AP. In applications of extracting TCM, studies have shown that extraction time could be reduced and protein activity could be maintained when extracted by microemulsion [29-31]. We have used microemulsion as a drug carrier to contain both water-soluble and fat-soluble components in Angelica and generated a new Angelica preparation with better efficacy and bioactivity [32].

In general, biocompatibility refers to the response capacity of biomaterials in dynamic and static changing process in vivo; a good biocompatibility means that the preparation is relatively stable in biological system, without any effects on clinical responses and/or toxicity of the main drugs [33]. Biocompatible microemulsion (BM) has various characteristics of biocompatibility that the four phases (water, oil, surfactant, and co-surfactant) are harmless to the organism and have no rejection reaction at the same time. Cholesterol is a derivative of cyclopentanoperhydrophenanthrene, which is similar to the fat regarding solubility and suitable for forming oil/water (O/W) microemulsion. As an essential component of cell membrane, with low toxicity, this compound is very suitable for preparing drug carrier system. Lecithin has strong surface and physiological activity, because of the fatty acid structure with strong lipophilic and the amino and phospholipid structures with strong hydrophilic of phosphatidylcholine (PC). The main component of Lecithin, a natural surfactant, has been provided a strong foundation in emulsifying ability by this amphoteric compound [34]. Bile salts (BS) have strong solubility for many drugs [35] and can accommodate more water-insoluble drug molecules after adding PC. The molar ratio of lecithin and bile salts is 1 : 3 (the physiological proportion of human bile) [36-38]; when lecithin is mixed with hydrogenated castor oil (RH-40) with the ratio of 1 : 1, the solubilizing power could be enhanced significantly.

The aim of the present study was to prepare a microemulsion with optimal formulation using mixed experimental design (a method of mixing a number of different materials into a stable substance), and use the microemulsion to prepare BMAP, in order to establish a new, more efficient and suitable extraction method that could overcome the inadequacies of the current AP preparations.

**Materials and Methods**

**Materials**

AP was purchased from Beijing Huamiao Traditional Chinese Pharmaceutical Engineering and Technology Developing Center (Beijing, China), identified as the dried aerial parts of *Andrographis paniculata* (Burm.f) nees, and stored in dark hermetically; andrographolide and dehydroandrographolide were purchased from National Institute for Food and Drug Control (Beijing, China); PC50 lecithin was purchased from Shanghai Taiwei Pharmaceutical Co. (Shanghai, China); cholesterol and isopropyl myristate were purchased from Sigma-Aldich Co. (Shanghai, China); pig bile salts was purchased from Beijing Shuangxuan Microbiological Media Products Factory (Beijing, China); polyoxyethylene hydrogenated castor oil was purchased from BASF Co. (Guangzhou, China); acetonitrile and methanol were chromatographically pure, which were purchased from Tianjin Yongda Chemical Agent Development Center (Tianjin, China); and all other chemicals and reagents were of analytical pure and used without further purification.

**Animals**

Twelve (6 for each sex) Japanese big-eared rabbits (*Oryctolagus cuniculus*) were obtained from Vital River Laboratories (Beijing, China) and housed under sterile condition with free access to common diet and tap water for 2 weeks before experiments. The relevant experiment protocols were approved by the Institutional Animal Care and Use Committee of Beijing University of Chinese Medicine.

**Preparation of BM**

We chose cholesterol and IPM as oil phase, bile salts/phosphatidylcholine as a surfactant, and RH-40 and ethanol as co-surfactants for preparing BM. Design Expert 17.0 was used for experimental design, as the testing of regression equation, the determination of the constant value and the most value, as well as other functions were integrated by this software. D-optimal design in mixture designs was selected to optimize the formulation of BM (Table 1). The final BM was prepared according to the optimized formula. First, a certain amount of lecithin was dissolved in RH-40 to make a uniform mixture, to which the mixed solution of cholesterol and IPM (proportional to the amount of 95%...
ethanol in the optimized formula) was added with stirring evenly. The BS dissolved in double distilled water was added into the aforementioned mixture slowly with continuous stirring by using a constant temperature magnetic stirrer until a transparent microemulsion was formed. After being appropriately diluted, the particle size and polydispersity index (PDI) of BM were measured by Malvern particle size analyzer (Malvern, Nano-zs, UK). The methods of regression analysis (backward) and cubic polynomial fitting were used to analyze the dynamic changes between the dependent variable (the particle size) and independent variables (all levels of the oil phase, aqueous phase, surfactant, and co-surfactant). Regression coefficients and constants were calculated before investigating the accuracy of regression equation with the indexes of fitting degree and the correlation coefficients and the optimal BM formulation was filtered by the method of analysis of variance.

**Determination of physicochemical property of the BM**

The droplet size of the BM was measured by Malvern particle size analyzer. The BM sample prepared according to the optimized formulation was appropriately diluted with double distilled water and filtered through a 0.45-μm microporous membrane in order to reduce the interference of particulate matter. The filtered BM sample was put into a standard quartz cuvette to measure the droplet with Malvern particle size analyzer, with wavelength of 658 nm, scan angle of 90°, and temperature of 25 °C. The data were then analyzed by using the software package provided by the manufacturer. Each BM sample was measured for six times and the mean value was presented.

The droplets of the BM were morphologically observed under a transmission electron microscope (TEM, JEM-1230, JEOL, Tokyo, Japan). The BM sample was diluted with double distilled water appropriately to avoid the aggregation of microparticles. When a liquid membrane formed directly, the BM sample was stained with 2% (W/W) phosphotungstic acid solution (pH 7) for 15 min and then dried at room temperature.

An appropriate amount of BM sample was centrifuged twice (10 min each) at 1 000 g at room temperature, while the same BM samples without centrifugation were kept at 0, 4, 25, and 50 °C for 3 months. Then the clarity and morphological changes of BM were observed by visual observation and absorbance detection at 500 nm wavelength using an ultraviolet spectrophotometer.

**BMAP preparation using different methods**

In our preliminary experiments, we compared extraction efficiencies of active ingredients in AP using BM or ethanol as extraction solvent. AP (20 g) was dissolved in 200 mL of BM, stirred evenly, and then extracted with ultrasonication for 30 min at room temperature, water bath for 30 min at 70 °C, or cold macerating for 4 days at room temperature, respectively. The filtrates of BMAP extracted by these methods were collected after decompressed filtration, respectively. The procedures percolating extraction were as follows: 20 g of AP was dissolved in 20 mL of BM and stirred evenly, protected from light for 1 h to make AP wet or swell sufficiently, and then transferred to a percolator. After tiny air bubbles were eliminated, 180 mL of BM was added to allow for dipping for 36 h (sealed), and the filtrate of BMAP was collected in the process of percolate extraction at room temperature. Ethanol extract of AP (the concentration of ethanol was 28.8%) was prepared with the same extraction methods.

**Determination of andrographolide and dehydroandrographolide**

The reference solutions of andrographolide and dehydroandrographolide were prepared in methanol to form the final concentration of 0.1 mg·mL⁻¹ separately. 2 mL of the BMAP or ethanol extract was dissolved in 6 mL of methanol, mixed with a vortex mixer for 120 s, and then centrifuged at 3 000 g for 10 min. The supernatants were filtered with 0.45-μm membrane filters and 20 μL was injected into HPLC system (Waters 2695/2996, USA) to quantify the contents of andrographolide and dehydroandrographolide. The HPLC system included a Waters 2690 equipped with a gradient controller, an automatic sample injector, and a 2996-photodiode array detector. Separation was performed on a C18 column (150 mm × 4 mm, I.D. 5 μm, DIKMA, China). The mobile phase consisted of acetonitrile (A) and water (B) at a flow rate of 1.0 mL·min⁻¹ with gradient elution as follows: 0–5 min, 22%–25% A; 5–30 min, 25%–30% A; and 30–50 min, 30%–40% A. The dual-wavelength was set at 225 and 254 nm, and the column was maintained at room temperature.

**Evaluation of bioavailability of the BMAP**

Japanese big-eared rabbits of 205–300 g (Vital River, China) were maintained in an air-conditioned animal quarter provided by the Laboratory Animal Center of Beijing University of Chinese Medicine (Beijing, China) at (22 ± 2) °C and relative humidity of 55% ± 5%, with free access to water and diet. The animals were acclimatized for 5 days, fasted for 12 h, with free access to water, prior to experiment, and randomly allocated into two groups (6 in each group). The rabbits were anesthetized with 20% urethane and treated with carotid artery catheterization. About 5 mL of baseline blood sample was collected via the carotid artery and stored in heparin-anticoagulated tube until analysis. One group was administered ethanol extracts of AP while the other group was administered BMAP by gavage at a dose of 20 g·kg⁻¹ (based on the calculated andrographolide concentration of 40 mg·kg⁻¹). The medicated blood samples (5 mL) were centrifuged at 4 ºC, plasma samples were obtained and stored at −20 ºC.
until analysis. An aliquot of plasma sample (100 μL) was mixed with 300 μL of absolute ethanol with a vortex mixer for 120 s and centrifuged at 1 000 g for 15 min. Then 20 μL of resulted solution was injected onto HPLC system for evaluating the oral bioavailability of the BMAP. Chromatography was performed using the method described above.

Statistical analysis
SPSS Version 20.0 for Windows (SPSS Inc. Chicago, IL, USA) was used for statistical analysis. The data are presented as means ± standard deviation (SD). Differences between groups were evaluated by post-hoc test and P < 0.05 was considered statistically significant.

Results and Discussion

The formula of the BM
After the type of surfactant, co-surfactant and the oil phase with the characteristics of good biocompatibility and biological activities were determined, the pseudo-ternary phase diagrams was used to screen the numerical boundary of the mixed surfactant, the oil phase and the aqueous phase for mixed design. The mixed surfactant (Smix) could be prepared when the $K_m$ values were 1 : 4, 1 : 2, 1 : 1, 2 : 1 and 4 : 1, and then mixed with oil phase, according to the volume proportion of 1 : 9, 2 : 8, 3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2, and 9 : 1, which was performed in double distilled water with stirring by a magnetic stirrer until a clear transparent microemulsion was formed. The amount of added double distilled water was recorded in order to determine the microemulsion-forming boundary points. According to the titration results, the limit values of each phase with different $K_m$ were obtained as follows: the surfactant ($X_1$): 0.060–0.300, co-surfactant ($X_2$): 0.030–0.050, an oil phase ($X_3$): 0.009–0.030 and the aqueous phase ($X_4$): 0.620–0.901.

Considering the impact of the ethanol content on solubilization effect, $K_m = 1 : 4$ mixed experimental design (Table 1) was chosen as the optimized value because of the bigger microemulsion-forming area and the ability to form more stable microemulsion [39].

According to Table 1, a certain amount of surfactant and the oil phase were mixed and stirred by a magnetic stirrer before slowly adding the co-surfactant and water until a homogeneous BM was formed, and the BM was then diluted before slowly adding the co-surfactant and water until a

<table>
<thead>
<tr>
<th>No.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>0.013</td>
<td>0.715</td>
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<td>2</td>
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<td>0.020</td>
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</tr>
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<td>0.018</td>
<td>0.820</td>
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<td>6</td>
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<td>0.040</td>
<td>0.030</td>
<td>0.870</td>
</tr>
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<td>14</td>
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<td>0.030</td>
<td>0.009</td>
<td>0.900</td>
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<td>15</td>
<td>0.270</td>
<td>0.050</td>
<td>0.030</td>
<td>0.650</td>
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<td>16</td>
<td>0.060</td>
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<td>19</td>
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<tr>
<td>20</td>
<td>0.290</td>
<td>0.030</td>
<td>0.030</td>
<td>0.650</td>
</tr>
</tbody>
</table>

Note: A: surfactant (0.060–0.300); B: co-surfactant (0.030–0.050); C: oil phase (0.009–0.030); D: aqueous phase (0.620–0.901)

An obvious trend of this effect curve is shown from the red region to the blue region (particle size changed from larger to smaller), indicating a great change in particle size that was impacted significantly by these factors. At the same time, the contour plot (the yellow area) projected by 3D shows that any change in the other two factors could be caused by any one of the three in the same contour line. Therefore, a significant interaction existed among surfactant, co-surfactant, and oil phase. Each index mentioned above was calculated by statistical analysis and the regression equation obtained as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4$$

The equations obtained by cubic polynomial fitting and the multivariate thrice multinomial obtained ultimately were

![Fig. 1 Effect curve of BM in different proportions](image-url)
The BM prepared according to the optimized formula was a clear and transparent yellow-brown homogeneous system, belonging to O/W type. The stratification and flocculation did not appear when the sample was kept at room temperature. No change was found in the appearance and the OD values maintained stable when the BM samples were kept at 0, 4, 25, and 50 ºC for 3 months. The results indicated that routine changes in the environmental temperature may not affect the physicochemical properties of the BM. But the sample became turbid, flocculated and layered when kept at 80 ºC for 24 h, indicating that higher temperature may destroy the microstructure of the BM to a certain extent, which was associated with the thermodynamic properties of BM.

**Active ingredients of the BMAP**

The BMAP containing a variety of chemical compositions was obtained by using cold macerating extraction when BM was used as solvent and AP as extracting target. The diterpene lactones, the major compounds in AP, have a wide range of biological activities, such as inducing differentiation of cells, antihepatotoxic, improving bile secretion and changing its physical properties. As the major diterpene lactones in AP, andrographolide and dehydroandrographolide have higher pharmacological activities than any other active ingredients of AP are used more in clinical practice; they were measured by HPLC in the present study. The BM sample remained clear and transparent after being centrifuged twice (10 min each time) at 1 000 g at room temperature. No change was found in the appearance of the BM sample prepared according to the optimized formula and interactions between these factors and indicators are shown in Table 2. The results indicated that the interaction among surfactant, co-surfactant and oil phase was highly significant ($P < 0.000 1$) and the regression equation could be used to estimate the values of each formula within the range of factors and levels. According to the minimum value of the equation, the minimum points (0.232, 0.031, and 0.013) were determined, and the optimal prescription of BM was surfactant: co-surfactant: oil phase: aqueous phase = 0.232 : 0.031 : 0.013 : 0.724. The BM was prepared under this optimum condition and the particle size and PDI of this BM were measured in order to investigate the physicochemical properties.

### Table 2 Variance analysis of optimal formula of BM

<table>
<thead>
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<th>Source</th>
<th>Sum of Squares</th>
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<th>Mean square</th>
<th>F Value</th>
<th>P-value</th>
<th>Prob &gt; F</th>
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<td>11 101.26</td>
<td>6.366 × 10^7</td>
<td>&lt; 0.000 1</td>
<td>Sig.</td>
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<td>Linear</td>
<td>65 162.97</td>
<td>3</td>
<td>21 720.99</td>
<td>6.366 × 10^7</td>
<td>&lt; 0.000 1</td>
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<tr>
<td>Mixture</td>
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<td></td>
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<td></td>
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<tr>
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<td>40.22</td>
<td>6.366 × 10^7</td>
<td>&lt; 0.000 1</td>
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<tr>
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<td>398.91</td>
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<td>398.91</td>
<td>6.366 × 10^7</td>
<td>&lt; 0.000 1</td>
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<td>AD</td>
<td>42.56</td>
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<td>42.56</td>
<td>6.366 × 10^7</td>
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<tr>
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As in Fig. 3, the contents of andrographolide and dehydroandrographolide in BMAP extracted by cold macerating extraction were higher than that extracted by other three methods. The contents of these two ingredients in BMAP were greater than that in ethanol extract, suggesting that BM was more suitable for extracting the diterpene lactones than ethanol and a higher extraction yield of andrographolide and dehydroandrographolide could be gained by cold macerating extraction.

In our experiment, many small peaks indicating other chemical compositions in AP were found on the baseline from HPLC, suggesting that BM can extract more fat-soluble and water-soluble components at the same time and play the role of biological trapping effect better, compared with ethanol extraction (Fig. 4).

**Pharmacokinetics of the BMAP**

The standard curve equation $Y = 1.8394X - 0.3068$ ($R^2 = 0.9924$) was calculated by linear regression over the range 0.1–2.3 μg mL$^{-1}$ where $Y$ and $X$ are peak area and plasma concentrations, respectively.

After oral administration of BMAP and ethanol extract of AP to rabbits, the plasma concentration-time profiles of andrographolide are presented in Fig. 5 (the content of dehydroandrographolide was too low to detect). It was found that the concentrations of andrographolide in BMAP were higher than that in ethanol extract almost at each time point and maintained a high blood concentration for a long time. The concentration of andrographolide in BMAP group (with three obvious absorption peaks at 30 min, 1.5 h, and 3 h) declined more slowly after reaching the peak at 3 h compared
Fig. 4 Representative HPLC chromatograms of microemulsion (A), reference substance of andrographolide and dehydroandrographolide (B), Ethanol extract (C), and BMAP (D). 1. Andrographolide 2. dehydroandrographolide

Fig. 5 Concentration-time curves of andrographolide in BMAP and the ethanol extract

Table 3 Pharmacokinetic parameters of BMAP and ethanol extract of AP

<table>
<thead>
<tr>
<th></th>
<th>$AUC_{0-\infty}/(\mu g\cdot mL^{-1}\cdot h^{-1})$</th>
<th>$AUC_{0-7}/(\mu g\cdot mL^{-1}\cdot h^{-1})$</th>
<th>$C_{\text{max}}/(\mu g\cdot mL^{-1})$</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$K$</th>
<th>$EDA_{0-\infty}$</th>
<th>$EDA_{0-7}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAP</td>
<td>2.267 ± 0.067 *</td>
<td>27.156 ± 0.63 **</td>
<td>0.301 ± 0.02</td>
<td>0.5 ± 0.02</td>
<td>0.009 ± 0.00</td>
<td>6.303</td>
<td>1.420</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>1.606 ± 0.084</td>
<td>4.308 ± 0.44</td>
<td>0.301 ± 0.05</td>
<td>3 ± 0.21</td>
<td>0.067 ± 0.04</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$ vs ethanol extract

To improve the solubility and the dissolution rate of poorly soluble drugs is an important way to improve the bioavailability of oral administration [44]. After entering the gastrointestinal tract, one part of the drug was mainly transported via intestinal epithelial cells into the portal vein, then entering into the systemic circulation after being metabolized by liver, and another part can be ingested into the lymphatic capillaries through the knot (PP) on intestinal mucosa and remitted to the thoracic duct before entering into the circulation through jugular vein [45]. In our experiment, andrographolide in BMAP can be absorbed by lymphatic after oral administration and contacted the epithelial cells of gastrointestinal directly after passing through the hydration layer of the gastrointestinal wall, which made this ingredient be absorbed faster.
Conclusion

In our study, cholesterol was used as oil phase, lecithin as surfactant and bile salts as co-surfactant. By combining with polyoxyethylene castor oil, a microemulsion with a good biocompatibility and low toxicity can be prepared. With the methods of mixed design and the mathematical model, the best ratio of the BM was screened and the BMAP with good biocompatibility was prepared successfully. With an average droplet size of 19.12 nm, the BM has good thermodynamic stability under different conditions. Compared with the ethanol extract of AP, the BMAP showed stronger drug potency with a higher blood concentration and better bioavailability after oral administration. In conclusion, some shortcomings in the current extraction methods of AP could be overcome by using BMAP which extracted comprehensively and thoroughly, with higher bioavailability.

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References

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