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Formulation, Characterization and Anti-Tumor Synergistic Evaluation of Nano Emulsion Formulation Containing Curcumin and Brucea Javanica Oil

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ABSTRACT

Objective: The aim of the study was to prepare and in more details improve antitumor effect by nano emulsion (NE) loaded with curcumin (CUR) and brucea javanica oil (BJO) together.

Materials and Methods: Solubility of CUR and BJO in various vehicles were conducted in a shaking incubator. NE was prepared by pseudo-ternary phase diagrams to obtain the concentration range of components. The physicochemical and biological characteristics of the NE was studied by size and zeta potential, morphologies, physical stability, *in vivo* antitumor activity and *in vivo* pharmacokinetics.

Key Findings Results: The combination of BJO and CUR in optimized NE formulation exhibited excellent stability. Besides, it showed a synergy antitumor effect and enhanced bioavailability.

Conclusions: The combination of BJO and CUR in optimized NE was successfully formulated. BJO used as the oil phase produced an antitumor synergistic effect with CUR was investigated by anti-tumor activity experiment. The dissolution rate and oral bioavailability were also improved compared with pure CUR.

INTRODUCTION

Brucea javanica oil [BJO] a complex mixture of fatty acids and fatty acid derivatives, is extracted from the ripe fruits of *Brucea javanica* [L.] Merr. [Simaroubaceae] ^[1,2]. It is reported that BJO has various ailments potent pharmacological activities such as tumor suppressive, anti-inflammatory and antimalarial activities ^[3-6]. It also has effects for clinical treatment of intermediate and advanced stage malignant tumors ^[7]. Reports have confirmed that BJO has the ability to ensure the safety of bone marrow hematopoietic stem cells, and increase the overall quality of life for patients undergoing tumor treatment ^[2].

Curcumin [CUR], a natural hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa*. It has a wide pharmacological profile that includes anti-oxidant and anti-microbial ^[8,9]. It also has tumor suppressive of cell lines, including breast, head, colon and etc ^[10-13]. Pharmacological studies showed CUR has a low toxicity, making it a promising drug for clinical treatment ^[14,15]. However, the low solubility and poor bioavailability of CUR had affected the dissolution rate, metabolism and permeability. Low solubility will then limit the therapeutic effects and clinic application ^[16]. Development a formulation that loading of two therapeutic agents has been a trend especially in the treatment of cancer diseases.^[17] In the field of cancer chemotherapy, it is rarely to have just a single agent that is treated in the disease ^[18]. CUR and BJO have played an important role in anti-tumor treatment and both of them have wide anti-tumor spectra and good therapeutic effects. Several investigations have confirmed the therapeutic superiority of combination treatment over the single drug treatment ^[19]. To the best our knowledge, there is no literature reported on the preparing both of these two drugs in the form of NE.

The low solubility and poor bioavailability of the CUR and BJO have limited their therapeutic effects. The important thing in this article is selecting the suitable drug delivery. For the rate and extent of drug from carrier are important to achieve therapeutic local concentrations in a reasonable time frame and provide sustained pharmacological action^[20]. Over the past decade, there has been progress in the development of nanoemulsification drug technology. Nanoemulsion identified as a promising delivery systems had a nanoscale droplet diameter, relative high rate of drug release from oil droplets to aqueous media, high solubilisation properties^[21-26].

As an effective drug carrier of increasing the absorption and solubility of poorly water-soluble drugs, it has received increasing attention for good properties^[27-31]. Nano emulsions were composed with water, oil, surfactants and co-surfactants. NE is a thermodynamically stable formulation and possible to avoid hepatic first pass metabolism and to raise lymph directivity depending on the kind of oil phase; thus, NE also are capable of enhancing the skin delivery of both hydrophilic and lipophilic drugs. It's small droplet sizes prevented phase separation and provide better adherence to membranes^[32,33].

Nanoemulsion formulation needs a certain amount of oil. While, there were no reports on the BJO as the oil of NE formulation. The purpose of this study was focused on the nanoemulsion preparation by using BJO as oil phase. Then a NE formulation containing BJO and CUR (CUR-BJO-NE) was developed. We wished combination using of them could achieve synergistic antitumor effect and enhance oral bioavailability. Formulation was then evaluated by characterization, stability test and. Bioavailability. The characterization of nanoemulsion was conducted by it appearance of using transmission electron microscopy (TEM) and droplet size. Oral bioavailability study in rats was carried out to evaluate the absorption of CUR-BJO-NE compared with the CUR suspension (CUR powder dispersed in 0.4% CMC-Na solution). Finally, *in vivo* anti-tumor activity was carried out to estimate therapeutic potential of CUR-BJO-NE. With all these results we could get information about its stability and clinical.

MATERIALS AND METHODS

Materials

CUR was obtained from the Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). BJO was obtained from Yaoda Pharmaceutical Co. Ltd (Shenyang, China). Capryol 90 was obtained by Gattefosse (Shanghai, China). Ethyl Oleate was purchased from Aotai Chemical Ltd. (Jinan, China). Peanut oil was obtained from Yihai Jiali Food Marketing Ltd (Beijing, China). Polyoxyl 40 hydrogenated castor oil 40 (Cremophor RH 40) and Polyethylene glycol 400 (PEG400) was purchased from BASF Co., Ltd. (Germany) and Huadong chemical reagent factory (Tianjin, China), respectively. Tween 80 was purchased from Meilin Industry and Trade Co., Ltd (Tianjin, China). Sodium carboxymethylcellulose (CMC-Na) was purchased from Fuchen chemical reagent factory (Tianjin, China). All other chemicals and solvents were analytical reagent grade.

Animals

Male Wistar rats (250 ± 20 g) were provided by Vital River Laboratory Animal Center (Beijing, China). Kunming mice (18-22 g) were purchased from Laboratory Animal Centre of Hebei Medical University, (Shijiazhuang, China).

Cell lines

The mouse ascetic turnout cell line S180 cell line provided by the cell bank of Chinese academy of sciences, Beijing, China.

Preparation of NEs

Solubility study

The solubility of CUR and BJO in various vehicles were conducted and measured as follows. Firstly, 1 mL of selected vehicles was added to each centrifugal tube containing a quantitative of BJO. Then the centrifugal tubes containing the mixture were kept in a shaking incubator at 25 °C for 2 days to get equilibrium. The quality of mixing uniformity's degree was selected as the indicator of vehicles. Besides, excess amount of CUR were separately added to 1 mL of various oils and surfactants in the centrifugal tube, followed by mixing in a shaking incubator at 25 °C for 48 h. The solution was centrifuged at 10000 rpm for 10 min to remove the excess CUR, after which the concentration of CUR in the supernatant was measured by UV spectrophotometer after appropriate dilution with ethanol.

Construction of pseudo-ternary phase diagrams

Pseudo ternary phase diagrams were constructed in order to obtain the concentration range of components for the existing region of NE. Different weight ratios of oil phase, surfactants and co-surfactants were mixed together, and distilled water was added drop by drop to the mixture under proper magnetic stirring at room temperature until the mixture became clear at a certain point. The concentrations of the components were recorded in order to complete the pseudo ternary phase diagrams.

Preparation of NE formulations

CUR-BJO-NEs were prepared at various component ratios based on the results of pseudo ternary phase diagrams. BJO was used as an oil constituent and CUR was dissolved in the surfactant mixture under apparatus of ultrasonic cleaner. Certain amount of co-surfactants and the mixture of oil were added to the mixture drop by drop and the mixture was stirred at 25 °C under light

shielding. Then undissolved CUR was removed by 0.45 µm membrane. The NE formulation contains neither BJO nor CUR (Blank NE) and the NE formulation contains CUR only (CUR-NE) were also prepared in the same procedure for further study.

Characterization of NEs

Mean droplet size of NEs

The droplet size and zeta potential of the CUR-BJO-NE were measured by a photon correlation spectroscopy (PCS) using a NICOMP particle sizing system (CW 380, Santa Barbara, CA) at a fixed angle of 90 degrees at 25 °C. The droplet size of NE was evaluated using volume distribution. Polydispersity index (PI) which indicated the width of the size distribution.

TEM

The morphologies of the CUR-BJO-NE were observed by transmission electron microscopy (TEM) (JEM-100SX, JEOL, Japan). One drop of diluted NE sample was negatively stained by 2% phosphotungstic acid (PTA) and placed on copper grids followed by drying at room temperature before examination.

Centrifugation

Physical stability of the selected vehicles as well as drug-loaded NEs was tested by centrifugation at 5000 rpm for 10 min.

HPLC analysis

All samples were analyzed by HPLC that consisted of LC-20A liquid chromatogram and equipped with a SPD-20A UV/VIS detector (Shimadzu, Kyoto, Japan). A reversed phase C₁₈ column (5 µm, 250 mm × 4.6 mm) in conjunction with a security guard column was used in this process at 430 nm. The mobile phase was a mixture of acetonitrile: H₂O (containing 5.0% acetic acid) (60:40, v/v) at a flow rate of 1.0 mL/min.

Pharmacokinetics study of CUR-BJO-NE in rats

Wistar rats were randomly divided into 2 groups and each with 5 rats. This animal experiment was approved by the Institutional Animal Care and Use Committee and was in compliance with all regulatory guidelines. The rats were fasted overnight before experiment with free access to water. CUR suspension (1000 mg/kg of body weight, pure CUR powder dispersed in 0.4% CMC-Na solution) and CUR-BJO-NE (400 mg/kg of body weight) were given to rats by intragastric administration. 0.5 mL of blood was obtained from ophthalmic vein of rats at predetermined time intervals and centrifuged at 4000 rpm for 10 min. Then the blood samples were stored at -20°C before the analysis. 0.2 ml plasma was added with 0.6 ml ethanol and it was for 5 min vortex. Then the mixture was centrifuged at 4000 rpm for 10 min. The concentration of CUR in the supernatant was determined by HPLC.

In vivo antitumor activity

The CUR-BJO-NE against mouse model bearing the S180 cell line were established and compared with CUR-NE *in vivo* antitumor activities. The experiments were designed as follows: tumor-bearing mice were inoculated intraperitoneally with S180 cell line (mouse sarcoma cells) and the ascites was formed in about seven days. Then the ascites was extracted and washed with PBS and adjusted to cell suspensions in the serum free medium at cell concentration of 2×10^7 cells/ml and 200 µL of the mixture was implanted subcutaneously into the flank of nude mice to establish tumor xenograft. The tumor mouse model then was established. The mice were then randomized to four groups with accurately according the weight of each mouse. The treatment was started with 10 mice in each group.

The first two groups were intraperitoneally given CUR-BJO-NE and CUR-NE at the same dose of 50 mg/kg, respectively. Blank NE as a negative control group at the same volume was given in the same way. Cisplatin injection were given as a positive treatment group at a dose of 1 mg/kg. All of these groups received administration once a day for 14 days. After 14 days of administration, all mice were killed by cervical dislocation, and the tumors of the mice were collected and weighed to calculate the tumor growth inhibition rate (TGI) by the formula:

$$\text{TGI [\%]} = [W_c - W_t] / W_c \times 100\%.$$

W_c denoted the mean tumor weight of the negative control group and W_t expressed mean tumor weight the treated groups.

Statistical analysis

All the experiments in the study were performed at least three times and the data were expressed as the mean ± SD. A two-tailed unpaired Student's t-test was performed at $p < 0.05$.

RESULTS

Solubility study

BJO could form a homogeneous solution with Capryol 90, Cremophor RH40, Labrasol, Tween 80 and PEG400. However, it was immiscible in oleic acid ethyl ester, Transcutol HP and ethanol. The saturated solubility of CUR in various vehicles was shown

in **Table 1**. Among the various oily phases that were screened, Capryol 90 provided the highest solubility of CUR. Solubility of CUR in Cremophor RH40 was the highest among the surfactants, and then followed by Labrasol. Transcutol HP showed a better solubility for CUR, BJO doesn't dissolve in it. Then PEG400 was chosen as the co-surfactant for further study.

Table 1. Solubility of CUR in various vehicles at 25 °C saturated for 48 h.

	Vehicles	Solubility of CUR [mg/ml]
Oil	Ethyl oleate	0.65 ± 0.08
	Capryol 90	9.89 ± 0.18
	Peanut oil	0.17 ± 0.01
	Cremophor RH40	103.94 ± 0.37
Surfactant	Tween 80	2.18 ± 0.08
	Labrasol	8.42 ± 0.19
	Ethanol	6.33 ± 0.36
Co-surfactant	Transcutol HP	156.30 ± 0.11
	PEG400	16.52 ± 0.13

Construction of pseudo-ternary phase diagrams

In the preliminary results, the emulsifying effect was not very well when Cremophor RH40 and Labrasol was selected alone as a surfactant, while the combination use of them two could achieve a better effect. PEG400 was served as the co-surfactant, and BJO and Capryol 90 were chosen as oil phase. Cremophor RH40 and Labrasol were mixed at the optimized ratio of 3:1. Capryol 90 and BJO were mixed at 1:1 (w/w). Then the mixtures of prepared above were mixed with the oil phase to give the different mass ratios (Km) of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9. The pseudo ternary phase diagram was exhibited in **Figure 1**.

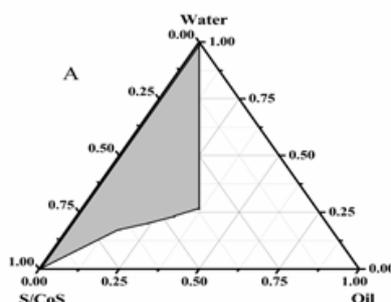


Figure 1. Pseudo ternary phase diagram composed of BJO and Capryol 90 (1:1, w/w) as oil phase, Labrasol and Cremophor RH40 mixed at the ratio of 1:3 (w/w).

The composition of the final formulation was selected inside the NE region. Considering the higher capacity of BJO loading, the final composition consist of 15% BJO, 15% Capryol 90, 56% surfactant mixture (Cremophor RH40/Labrasol, 3:1 w/w), 14% PEG400 was selected.

Characterization of CUR-BJO-NE

TEM Morphology of the optimized CUR-BJO-NE was showed in **Figure 2**. NE appeared to be spherical in shape and had a uniform size. The average particle size was around 51.5 nm.

The physical stability of CUR-BJO-NE was also evaluated. After centrifugation, the samples did not show any changes in appearance, homogeneity and no phase separation or breaking or drug precipitation was exhibited. This showed the formulation was physically stable after centrifugation.

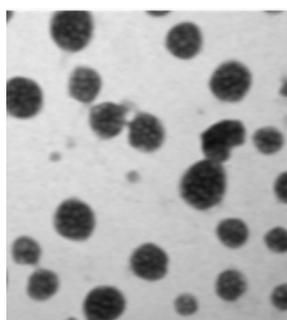


Figure 2. Transmission electron microscopy image of CUR-BJO-NE.

In vivo pharmacokinetic study

The CUR pharmacokinetic study in CUR-BJO-NE were evaluated and compared with CUR suspension. As shown in **Figure 3**, the plasma concentration of NE was higher than that of CUR suspension at each time point [$p < 0.05$], in spite of the oral dosage of CUR suspension is 2.5 times higher compared with NE formulation. The main pharmacokinetic parameters are summarized in **Table 2**. The maximum plasma concentration [C_{max}] of CUR-BJO-NE was enhanced about 2-fold compared to the CUR suspension. The AUC in NE increased 5.2-fold compared with that of CUR suspension [534.00 min-mg/L vs. 272.14 min-mg/L, $p < 0.05$]. In the case of oral dosage of CUR-BJO-NE being far lower than that of CUR suspension, there was still a high increase in parameters of C_{max} and AUC.

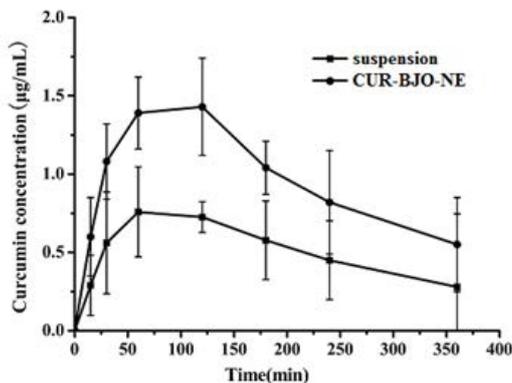


Figure 3. Mean plasma concentration time profiles of oral administration of CUR-BJO-NE and suspension to rats [mean SD, n=5].

Table 2. Main pharmacokinetics parameters of CUR-BJO-NE and suspension.

Parameters	CUR-BJO-NE	Suspension
$T_{1/2}$ [min]	215.29 ± 87.45	186.50 ± 84.43
C_{max} [mg/L]	1.52 ± 0.41	0.84 ± 0.11
T_{max} [min]	78.00 ± 40.25	84.00 ± 32.86
AUC [min-mg/L]	534.00 ± 162.25	272.14 ± 88.33
MRT [min]	334.61 ± 114.10	302.92 ± 108.02

$T_{1/2}$: half-time; MRT: Mean residence time AUC: Area Under The Curve; MRT: Mean residence time.

In vivo antitumor activity

The changes of mean tumor weight and tumor inhibition rate [TGI] in mice were assessed and shown in **Table 3**. After 14 days therapy, the average tumor weight were 1.79g for blank NE, 0.66 g for cisplatin positive group, 0.89g for group giving CUR-BJO-NE, 0.99 g for CUR-NE.

The tumor growth rate was highest in blank NE (negative control group), while the highest tumor inhibition rate was found in the positive control group. Compared with the group of blank NE, both of the two groups had an effective inhibition growth to tumor cells. CUR-BJO-NE had a higher inhibition of tumor growth at a TGI of 50.26% than that of CUR-NE with a TGI of 44.88%.

The value of initial body weight (IBW)/final body weight (FBW) was selected as value index to assess toxicities. It was regarded as no toxic reaction when IBW/FBW was greater than 0.8. The data showed in **Table 3** meant all the formulation in the effect dose level.

Table 3. Tumor growth inhibition of various formulations in tumor-bearing S180 cells mice.

Group	Weight [g]			Tumor weight [g]	TGI (%)
	IBW	FBW	IBW/FBW		
Negative group	26.86 ± 1.66	26.73 ± 4.83	0.99 ± 0.15	1.79 ± 0.60	
Positive group	25.61 ± 2.50	21.63 ± 2.79	0.84 ± 0.04	0.66 ± 0.17	63.11
CUR-NE	26.01 ± 1.34	24.69 ± 2.68	0.95 ± 0.08	0.99 ± 0.28	44.88
CUR-BJO-NE	25.62 ± 2.21	26.23 ± 3.80	0.99 ± 0.07	0.89 ± 0.30	50.26

CUR-NE: NE containing only drug of CUR; CUR-BJO-NE: formulation prepared in this article.

DISCUSSION

The solubility studies were conducted in the aim of finding out appropriate oils and surfactants in NE with high drug-loading capacity both for CUR and BJO. The emulsification properties with other ingredients were also considered for the selection of vehicles [34,35]. Then PEG400 was selected as the co-surfactant instead of Transcutol HP, although Transcutol HP showed a better solubility than PEG400. The combination using of Cremophor RH40 and Labrasol as the surfactants was that they could achieved

a better emulsifying effect than they alone using. This might be associated with lower the oil–water interfacial tension more sufficiently [36]. The enhanced bioavailability was probably due to the combination of the following effects: Firstly, dissolution rate of CUR was significantly improved in CUR-BJO-NE, oral absorption could be increased when drug was kept as dissolved state in NE; Secondly, the use of surfactant could also reduce or inhibit P-glycoprotein on the effect of drug efflux effect; Simultaneously, the smaller droplet size enabled the NE droplets to escape from uptake and phagocytosis by the reticuloendothelial system and increased the circulation time of the drug. The enhanced dissolution rate might be related to the large surface area of NE and the use of surfactants in formulation.

The results *in vivo* antitumor activity showed that CUR-BJO-NE and CUR-NE had an effective inhibition growth to tumor cells compared with the group of blank NE. It indicated that both CUR and BJO had anti-tumor effect to S180 cell. This also investigated that BJO employed as an oil phase to solubilize CUR produced a synergistic anticancer effect. In conclusion, all the results obtained strongly supported the development of potential formulation of CUR-BJO-NE.

CONCLUSION

In this study, a NE system containing both BJO and CUR was successfully formulated for oral delivery. BJO used as the oil phase produced an antitumor synergistic effect with CUR was investigated by anti-tumor activity experiment. The CUR-BJO-NE showed stability in the experiment of centrifugation and water dilution. The dissolution rate and oral bioavailability were significantly improved compared with pure CUR. The current study confirmed the potential using of combination CUR and BJO in the treatment of cancer.

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