doi.org/10.36721/PJPS.2022.35.6.REG.1503-1512.1

Study on the controlled release and synergistic anti-oxidant activity *in vitro* and *ex vivo* of ligustrazine hydrochloride encapsulated into liposomes

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Abstract: Ligustrazine with good antioxidant activity is one of the main active components of chuanxiong. We designed ligustrazine hydrochloride-loaded liposomes (LTH-L) by the thin film dispersion method. The particle size and zeta potential of liposomes was 118±10.61nm and -39.3±3.7mV, entrapment efficiency (EE%) was 75.05±10.67%. *In vitro* permeation across the dialysis membrane, the release rate (R%) of ligustrazine hydrochloride (LTH) and LTH-L were reached 80% and 60%. *Ex Vivo* transdermal behavior experiment showed the R% of LTH and LTH-L were between 30%-40%, the R% of LTH-L was slightly lower, because liposomes played the role on the sustained and controlled release of LTH. In addition, LTH, LTH-L and BL reacted with 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution for two hours, the scavenge rates (SR%) were 55.06±2.73%, 11.3±0.03% and 37.25±1.12% respectively (P<0.001) and the SR% of LTH, LTH-L and BL reacted with H_2O_2 were 4.13±0.02%, 0.52±0.01% and 75.15±6.10%. The inhibit rate (IR%) of LTH, LTH-L and BL on malondialdehyde (MDA) in liver homogenate were 35.44±1.79%, 1.22±0.01% and 17.92±0.29% (P<0.001), the IR% were 30.82±0.93%, 1.7±0.01% and 25.19±0.60% (P<0.001) in anti-low density lipoprotein (LDL) oxidation experiments, perhaps LTH prepared into LTH-L can play a better antioxidant role.

Keywords: Ligustrazine hydrochloride, liposomes, *in vitro* and *ex vivo* release, antioxidant effect.

INTRODUCTION

Chuanxiong is a common herbaceous plant with the homology of medicine and food. Because it is beneficial for health, it can be eaten as a dietary supplement. The fresh leaves of chuanxiong can be used as edible materials, for example, it can be mixed with eggs (fig. 1A) and the roots of chuanxiong (fig. 1B) can be stewed with fish head, beef and others (Z. Chen *et al*., 2018) (fig. 1C), this have a curative effect on headache and dizziness and greatly improve the adaptability of patients. In addition, some effective components of chuanxiong can also as cosmetics to delay the oxidative aging of skin and whiten skin.

Oxidation is a chemical reaction that causes metals to rust and flowers to wither. Oxidation reaction can also make the human skin aging, produce stains and wrinkles. In the human body, oxidation can cause inflammation, which is closely related to the occurrence of cardiovascular diseases. What's more, oxidative stress can destroy DNA structures and lead to diseases such as cancer. So it is particularly important for effective components of chuanxiong as antioxidant drug used to prevent the body from oxidative damage and treat diseases.

Related studies have proved that ligustrazine (LT), one of the effective components extracted from rhizoma of chuanxiong, accounting for about 0.01%-0.02% of all effective components, the extraction operation is difficult and the cost is high, now it can be obtained by artificial synthesis (Xiaoxiao *et al*., 2020). LT has the effect of promoting blood circulation and removing blood stasis (Beibei Fu, 2022), anti-platelet aggregation (L. Li *et al*., 2019)，treating stroke and slowing down the degree of brain injury (Allison, Josse, Gabriel, Klentrou, & Ditor, 2017). In addition, LT has strong antioxidant effect and can inhibit the generation of melanin, which may be used as a whitening ingredient. In melanin formation, tyrosine generates dopa with the participation of active oxygen and tyrosinase and further reacts to produce melanin. LT may inhibit the production of melanin by scavenging oxygen free radicals to hinder the generation of dopa based on its antioxidant activity.

LT insoluble in water, which can stimulate to gastrointestinal tract and muscles, is often made into ligustrazine hydrochloride (LTH) (fig. 1D) for clinical (Ge *et al*., 2020). LTH has strong antioxidant activity which can be explored and certified by characteristic antioxidant tests, such as scavenging DPPH free radical (DPPH·) (Alina *et al*., 2021) and H_2O_2 (Biswajita *et al*., 2021; Jeong, Bbeum, Hyo-Sun, In-Sook, & Joong, 2017). Furthermore, it can also inhibit lipid peroxidation to produce the MDA. At present, LTH has been prepared

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into various dosage forms. The commonly used dosage forms in clinic are injection and tablet. The novel preparation of LTH such as liposomes (Amr Selim Abu Lila, 2017), microcapsules and hydrogels have become the research hotspot (Heng, 2018). The optimization of these dosage forms maximizes the function of LTH and decreases side pharmacological effects.

In order to better study the antioxidant activity of LTH and better apply it to treat diseases, we encapsulate LTH into liposomes (LTH-L). The LTH-L can play different roles according to different routes of administration such as intravenously injected, orally administration or transdermal administration. Intravenous injection is the main mode of drug administration at present. However, with the deepening of the research, some achievements have been made in the research on oral and transdermal administration. However, liposomes are unstable, which is unfavourable for oral administration. The percutaneous approach can not only improve the therapeutic effect but also have no first-pass effect in liver. In this article, the main pharmacological effects were discussed by drug release and anti-oxidation experiment *in vitro* and *ex vivo*.

Fig. 1: LT as the effective component of chuanxiong with homology of medicine and food. (A) Whole grass of chuanxiong. (B) Root of chuanxiong. (C) Cooking with chuanxiong. (D) Structural formula of LTH.

MATERIALS AND METHODS

Materials

LTH (purity 99%), phosphate buffer solution (PBS, accurately weigh 7.90g of KH_2PO_4 and 34.0g of NaOH solid, added water to 5L, pH 7.4). Soybean phospholipids and cholesterol were purchased from Shanghai Jinsong Industry Corp., Ltd (Shanghai, China). 2, 2-Diphenyl1-picrylhydrazyl (DPPH) and 2-thiobarbituric acid (TBA) were provided by Shanghai yuanye biology science and technology Corp., Ltd (Shanghai, China) and Hydrogen Peroxide (H_2O_2) was provided by Shanghai suyi chemical reagent Corp., Ltd (Shanghai, China). $FeSO_4{\cdot}7H_2O$ was purchased from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China). Trichloroacetic acid (TCA) was obtained from Damao Chemical Reagent Factory (Tianjin, China). Healthy Kuming mice (male) were purchased from the Animal Experimental Center of Anhui University of Chinese Medicine (Hefei, China).

Methods

Drawing of LTH standard curve

Weighed 0.02g of LTH in a 100mL volumetric flask, added PBS to constant volume and dissolved completely by ultrasound to obtain 200ug/mL original solution. Pipette 1.0, 1.5, 2, 2.5, 3.0, 3.5mL original solutions into a 50mL volumetric flask and added PBS to constant volume, then shook it well and at least three parallel experiments were conducted on each point. PBS was used as a blank control to measure the absorbance of LTH with different concentrations at 295nm.

Preparation of LTH-L and blank liposomes (BL)

Liposomes were prepared by film dispersion method (Huang *et al*., 2019), weighed 0.300g of phospholipid and 0.100g of cholesterol into a round-bottom flask accurately and added 5mL absolute ethyl alcohol, which was completely dissolved in an ultrasonic water bath (70°C) instrument. Then let ethyl alcohol evaporated absolutely. At the same time, the round-bottom flask was rotated to form the liquid film sticking onto the wall of flask via rotary evaporator. In addition, prepared 100ml LTH solution with concentration of 0.6mg/ml with PBS solution, and put 10mL of it into the above film-forming flask and ultrasonic mixing until it was completely hydrated. LTH-L was obtained after being filtered 3 times across the microporous membrane with the pore diameter of 0.22μm for later use. In the same way as above, 10ml of PBS instead of 10ml of LTH solution was used to prepare BL.

Measure the zeta potential, particle size and entrapment efficiency (EE%) of LTH-L

The zeta potential and particle size of LTH-L were measured by a transmission electron microscope and a particle size analyser (Malvern Instruments Ltd., Malvern, UK). EE% was determined as follows: Added 1mL of LTH-L in a 10mL volumetric flask and set volume to the scale with PBS, shook and took out 3 mL of sample, centrifuged at 4000 rotate per minute (rpm) for 10 minute, took the supernatant liquid and measured the absorbance at the wavelength of 295nm. The drug concentration out of liposomes (C_o) can be calculated according to the standard curve. Took out another 1mL of LTH-L in a 10mL volumetric flask, added ethanol to a constant volume, ultrasonically dissolved liposome membrane, took out the supernatant liquid after centrifuging at 4000rpm for 10 minute and measured the absorbance at a wavelength of 295nm. The total concentration of drug (C_t) can be calculated according to the standard curve. EE% was calculated by formula (1), with higher encapsulation efficiency, the quality of liposomes would be better.

$$EE\% = \frac{[C_t - C_o]}{C_t} \times 100\% \quad (1)$$

Release rate of LTH and LTH-L across dialysis membrane in vitro

Measured volume of diffusion wells and filled it with PBS. Cut out dialysis membrane (MD34, MW range: 8000-14,000) with suitable size, spread the dialysis membrane

onto the receiving pool, pressed the dialysis membrane tightly with spring clips and pay attention to the fact that there is no air bubble between the receiving pool and dialysis membrane. Placed these diffusion wells on a constant temperature magnetic stirrer and the temperature was set at 37 °C. Added 1mL of PBS, LTH, BL and LTH-L into the supplying pool and took out 2mL of samples from receiving pool at the time of 5min, 10min, 20min, 30min, 60min, 120min, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h, 11h, 12h, 24h, 36h, 48h, 60h, 72h and 84h. Then filled it up with 2mL of PBS, measured the absorbance of the samples at the wavelength of 295nm, recorded the data and according to the standard curve to calculate the concentration, then got the R% according to the following formula (2) and drew the release curve with R% and time.

$$R(\%) = \frac{C_n \times V_n + \sum_{i=1}^{n-1} C_i \times V_i}{Q_t} \quad (2)$$

where "C_n" is the drug concentration of the dissolution medium at the nth sampling time point, "C_i" is the drug concentration at the n-1st sampling time in the sample, "V_n" and "V_i" are the volume of diffusion cell and the volume of the sample taken out and "Q_t" is the theoretical drug amount.

Release rate of LTH and LTH-L across the mouse skin ex Vivo

We ordered male mice with similar weight in advance, shaved the hair on the back of rats with a shaving machine and injected 0.2 mL of 20% urethane per 20 grams of body weight. Continued to use depilatory cream to remove back hair of mice completely, washed away the depilatory cream on the back with normal saline, peeled skin with scissors and removed excess subcutaneous fat with tweezers (fig. 2). All animal experiments were operated according to the guidelines approved by the ethics committee of Anhui University of Chinese Medicine (Hefei, China). The next operation steps were the same as dialysis membrane *in vitro*, except that the dialysis membrane was replaced with the back skin of the mice. And samples were taken at the specified time, measured the absorbance at the wavelength of 295 nm and got the R% and drew the release curve with R% and time.

DPPH free radical scavenging experiment in vitro

The scavenge effect of LTH on DPPH was determined by colorimetric (W. Chen *et al*., 2019; Huang, Su, Zheng, & Tan, 2017; Makoto, Yoshihito, Gen-Ichi, Keizo, & Ichiro, 2017), prepared 0.075, 0.10, 0.125, 0.25, 0.50 mg/mL of LTH in ethyl alcohol solution, set blank group (A_0: 2 mL PBS and 1 mL DPPH), control group (A_c: 2mL sample and 1mL PBS) and sample group (A_s: 2mL sample and 1mL DPPH).

Diluted 0.6mg/mL of LTH-L to 0.1 mg/mL, took out each 2mL of 0.1mg/mL LTH-L and five different concentrations of LTH solution, put them into 5mL centrifuge tubes separately, then took 1mL of DPPH solution (0.08mg/mL), added to the above centrifuge tubes and blend evenly, reacted for 0.5, 1.0, 1.5, 2.0 and 2.5h in the dark. Measured the absorbance at 517nm, recorded the data and calculated the scavenge rate (SR%) of LTH and LTH-L on DPPH• according to the following formula (3).

$$SR\% = \frac{A_0 - (A_s - A_c)}{A_0} \times 100\% \quad (3)$$

H_2O_2 scavenging experiment in vitro

To prepared H_2O_2 solution: Measured 0.2 mL of H_2O_2 in a 50ml volumetric flask, used distilled water to constant volume and prepared five different concentrations of LTH solution with PBS, which were 1.5, 2.0, 2.5, 3.0 and 3.5 mg/mL respectively, and diluted 0.6mg/mL of LTH-L to 0.09 mg/mL for later use. Set blank group (A_0: 0.6mL PBS and 1.8mL H_2O_2), sample group (A_s: 0.6mL LTH solution or LTH-L and 1.8mL H_2O_2) and control group (A_c: 0.6mL LTH solution and 1.8mL PBS). Evenly mixed, after 10 minutes, measured the absorbance at the wavelength of 230nm and calculated the SR% according to formula (4).

$$SR\% = \left(1 - \frac{A_s - A_c}{A_0}\right) \times 100\% \quad (4)$$

Inhibitory effect of LTH and LTH-L on MDA production in liver homogenate ex vivo

The liver of mice was taken out quickly after being anesthetized, rinsed repeatedly in cold physiological saline at 4℃, washed away blood stains, sucked dry with filter paper, weighed, added 9 times the weight of cold physiological saline (added in three times) and homogenized. Centrifuged at 4000rpm/min for 15 minutes, took the supernatant and made it into 10% liver homogenate and stored it in the refrigerator at 4℃ for later use, made 10mmol/L of $FeSO_4$ solution with distilled water. Set blank group (A_b: 1mL of 10% liver homogenate and 200μL of normal saline), model group (A_m: 1mL of 10% liver homogenate and 100μL of $FeSO_4$ and 100μL of normal saline), sample group (A_s: 1mL of 10% liver homogenate and 100μL of $FeSO_4$ and 100μL of LTH solution or LTH-L) and LTH solution was divided into five concentration respectively (0.025, 0.0375, 0.050, 0.0725 and 0.100 mg/mL). Each group needed to be mixed evenly. After mixed, the groups oscillated in a constant temperature shaker at 37°C for 1.5h. Then added 3mL of TBA working solution, which was 0.67% TBA mixed with of 10% TCA solution, mixed well, sealed the plastic wrap at the nozzle, water bath at 95°C for 40 minutes, cooled with running water, centrifuged at 4000rpm/min for 8 minutes and suck the supernatant. The

absorbance was measured at 532nm zeroed with normal saline by ultraviolet spectrophotometer. The inhibit rate (IR%) of MDA production was calculated according to the following formula (5).

$$IR\% = \frac{A_m - A_s}{A_m - A_b} \times 100\% \quad (5)$$

Inhibitory effect of LTH and LTH-L on MDA production due to low density lipoprotein (LDL) being oxidized In vivo

According to the improved precipitation method, LDL application solution was obtained from the mouse serum. The fresh blood of the mice was taken out, allowed it to coagulate naturally and centrifuged at 1000rpm for 10 minutes to obtain serum. Added 1mL of heparin citrate buffer solution to every 100mL of serum (prepared 0.064mol/L trisodium citrate with 20mL of 5 mol/L HCL, added 10mg of heparin and adjusted the pH value to 5.04). After mixed, stand at room temperature for 10 minutes, centrifuged at 1000 rpm for 10 minutes, adjusted the final pH value to 5.1, collected the precipitate and weighed it. Suspended the precipitate with 2 times of serum volume of high-salt phosphate buffer (pH 7.4), dissolved LDL precipitate and dialyzed at 4°C for 24 hours to obtain LDL extract. According to the pre-experiment, the LDL extract could be diluted by a certain multiple.

The experiment was divided into three groups: Blank group (1ml of LDL extract and 1.2mL of PBS), model group (1ml of LDL extract and 0.2mL of $FeSO_4$ and 1mL of PBS), sample group (1ml of LDL extract and 0.2mL of $FeSO_4$ and 1mL of LTH solution or LTH-L) and five different concentrations of LTH solution were tested (0.005, 0.01, 0.0125, 0.025, 0.05mg/mL). The function of $FeSO_4$ (10mmol/mL) was used to oxidize LDL to produce MDA. Next, incubated the groups at 37°C for 3h and then added 0.1 of mL EDTA-Na_2 to stop the reaction. Took 0.3mL of each reaction suspension, added 2.5mL of 20% TCA solution and then added 1.0mL of 0.67% TBA and mixed well. Each group was soaked in boiling water for 30 minutes, cooled to room temperature with tap water and centrifuged at 3000rpm/min for 10 minutes. The absorbance of supernatant was measured at 532nm wavelength and MDA content was calculated to represent the degree of LDL being oxidized. The IR% of MDA production due to LDL being oxidized was calculated according to the following formula (6).

$$IR\% = \frac{A_s - A_b}{A_s - A_m} \times 100\% \quad (6)$$

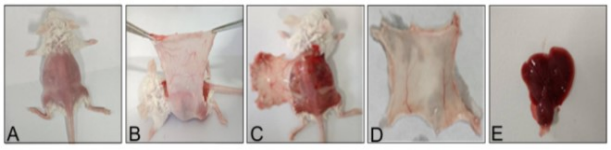


Fig. 2: The process of removing back skin and liver of mice. (A) Mice with back hair removed. (B) The outer side of the mice back skin. (C) The inner side of the mice back skin. (D) Remove the back skin of the mouse. (E) The liver of mouse.

STATISTICAL ANALYSIS

All the results obtained were expressed as mean ± standard deviation (SD) and computed by MS Excel Version, 2010. The statistical analyses were performed using SPSS Software 26.0 (IBM, Armonk, NY, USA) by an analysis of variance (ANOVA). The release curves of the drugs were analysed by using Origin software 2019b (OriginLab Corp, Northampton, MA, USA).

RESULTS

Standard curve of LTH

The regression equation obtained was A=0.0367*C+0.0009 (R^2=0.9999). It found that the absorbance of LTH was linearly correlated with the concentration in the range of 3.0μg/mL-15.0μg/mL and selected the detection wavelength of 295nm with high sensitivity and good specificity.

Results of particle size, zeta potential and EE% of LTH-L

Under the electron microscope, LTH-L was a spherical structure with a uniform particle size (fig. 3). After repeated operations for three times, the zeta potential of liposomes was negative with the value of -39.3±3.7 mV, the average diameter of liposomes was 118±10.61nm and the EE% of LTH-L was 75.05±10.67%. After 2 weeks' storage in the dark, the above indexes had no obvious changes; the particle size and EE% of LTH-L were 114±11.66nm and 73.14±9.87%.

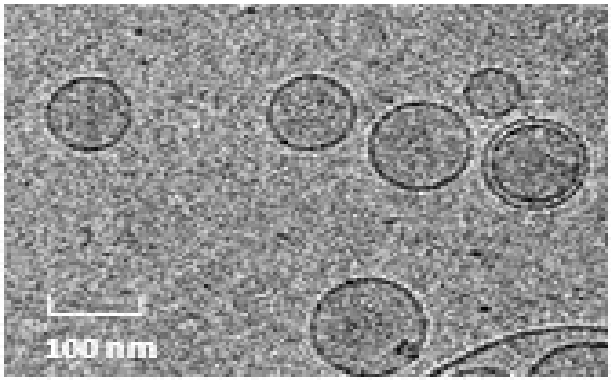


Fig. 3: Electron microscopic picture of LTH-L.

Release rate across dialysis membrane and transdermal experiments of LTH and LTH-L

Compared the R% across the dialysis membrane and mouse skin of LTH and LTH-L, which showed the permeability quantities of LTH was higher than that of LTH-L (fig. 4). Observed the diffusion curve and use origin software to fit the curve and get the appropriate

kinetic release model. We simulated the zero-order and first-order kinetic release models and the results were showed in table 1. The result showed that zero-order was not suitable and the first-order was the most suitable that the R^2 was greater than 0.9 (table 1), the fitting curve was shown in the below (fig. 5).

The permeability of drugs via mouse skin was less than that of dialysis membrane. The R% of LTH-L across the dialysis membrane was always greater than that of the transdermal permeation at the same time point and even reached two times that of the transdermal permeation. In the transdermal release process, within the corresponding time, the transdermal drug dosage of LTH was always higher than that of LTH-L.

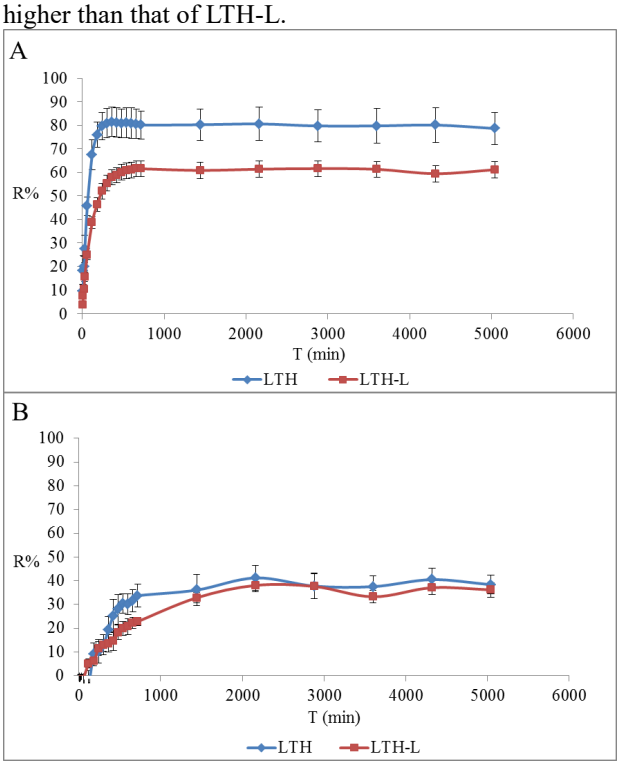


Fig. 4: Release curve of LTH and LTH-L. (A) R% of LTH and LTH-L across dialysis membrane. (B) R% of LTH and LTH-L across mouse skin.

Table 1: Zero-order model and first-order model fitting of diffusion curves for LTH and LTH-L.

Classify	Group	Model	Fitting equation	R^2
Dialysis membrane diffusion	LTH	Zero-order	y=59.78919+0.00643x	0.15593
		First-order	$y=80.64504(1-e^{-0.01504x})$	0.99320
	LTH-L	Zero-order	y=40.25761+0.0065x	0.22974
		First-order	$y=61.00417(1-e^{-0.00848x})$	0.99749
Transdermal diffusion	LTH	Zero-order	y=40.61612+0.00826x	0.48784
		First-order	$y=39.99217(1-e^{-0.00193x})$	0.92330
	LTH-L	Zero-order	y=8.91398+0.00763x	0.67844
		First-order	$y=37.09311(1-e^{-0.00136x})$	0.98713

The results of scavenging DPPH

The reaction mechanism was in fig. 6A and the reaction color map of LTH was in fig. 6B. At the same time, the LTH-L was also operated in the same way to obtain the following data and the reaction color map was in fig. 6C.

LTH had certain antioxidant activity, the SR% of LTH on DPPH• increased with the increase of LTH concentration, which indicated that its antioxidant activity was enhanced at a high concentration. In addition, the SR% of LTH on DPPH• gradually increased with the increase the reaction time. Under the appropriate concentration and reaction time, the antioxidant activity tended to be optimal. According to the scavenging curve, the longer reaction time with DPPH solution was, the greater the SR% value was. Within the same reaction time, with the increase of concentration, the SR% of LTH on DPPH• was more obvious (fig. 7A and 7B).

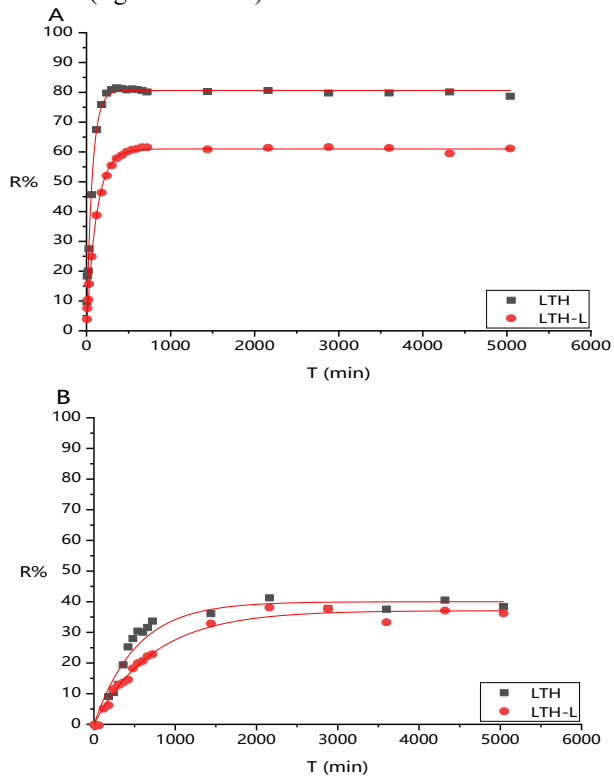


Fig. 5: Fitting of release curve by Origin software 2019b (OriginLab Corp, Northampton, MA, USA). (A) Fitting of diffusion curve across the dialysis membrane by first-order model. (B) Fitting of transdermal diffusion curve by first-order model.

It was found that the same concentration of LTH and LTH-L (0.1mg/mL) reacted with DPPH• for 2 hours later, their SR% were 55.06±2.73% and 11.3±0.03% respectively, LTH's scavenging efficiency on DPPH• was almost three times that of LTH-L and showed that the difference between LTH and LTH-L was statistically significant (P<0.001).

The results of scavenging hydrogen peroxide (H_2O_2)

The results showed that LTH had a good SR% on H_2O_2 (fig. 7C). With the increased of LTH concentration, its SR % on H_2O_2 gradually increased in a certain range. In this

experiment, the maximum SR% of H_2O_2 can reach 50%, and the SR% of LTH and LTH-L (0.09mg/mL) on H_2O_2 were 4.13±0.02% and 0.52±0.01%.

Inhibitory effect of LTH and LHT-L on MDA production in liver

Five different concentrations range from 0.0375 to 0.100mg/mL of LTH all could inhibit the formation of MDA in liver homogenate. The higher the concentration of LTH, the more obvious the inhibitory effect on the formation of MDA, which had a certain dose-effect relationship (fig. 7D). The IR% of LTH and LTH-L (0.0725mg/mL) on MDA production in liver homogenate was 35.44±1.79% and 1.22±0.01%. It found that the IR% of LTH-L on MDA was very small, only one seventeenth of that of LTH, the difference was statistically significant (P<0.001) (fig. 9), but LTH-L could also played an antioxidant role *Ex Vivo*. The reaction in liver homogenate simulated the anti-oxidation *in vivo*, which was different from the anti-oxidation property *in vitro*. The internal environment of an organism is complex and the drug reaction may not be so sensitive. Therefore, the IR% of LTH-L on MDA was lower than that of LTH. On the other hand, drugs was encapsulated in liposomes, which played a role of sustained and controlled release *in vivo* and the drug would be released slowly, so the antioxidant activity of LTH-L was different from that of LTH at that time.

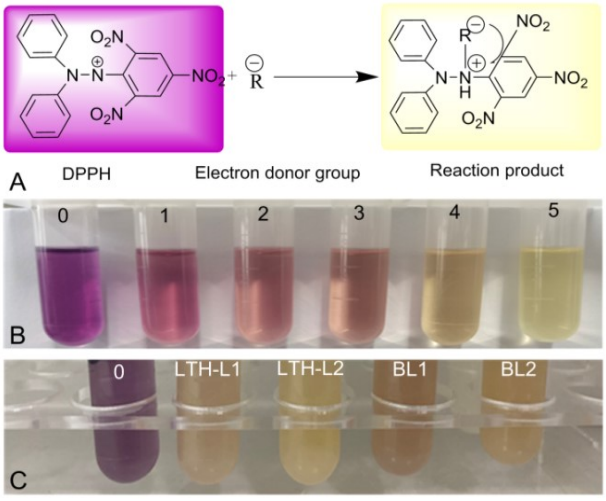
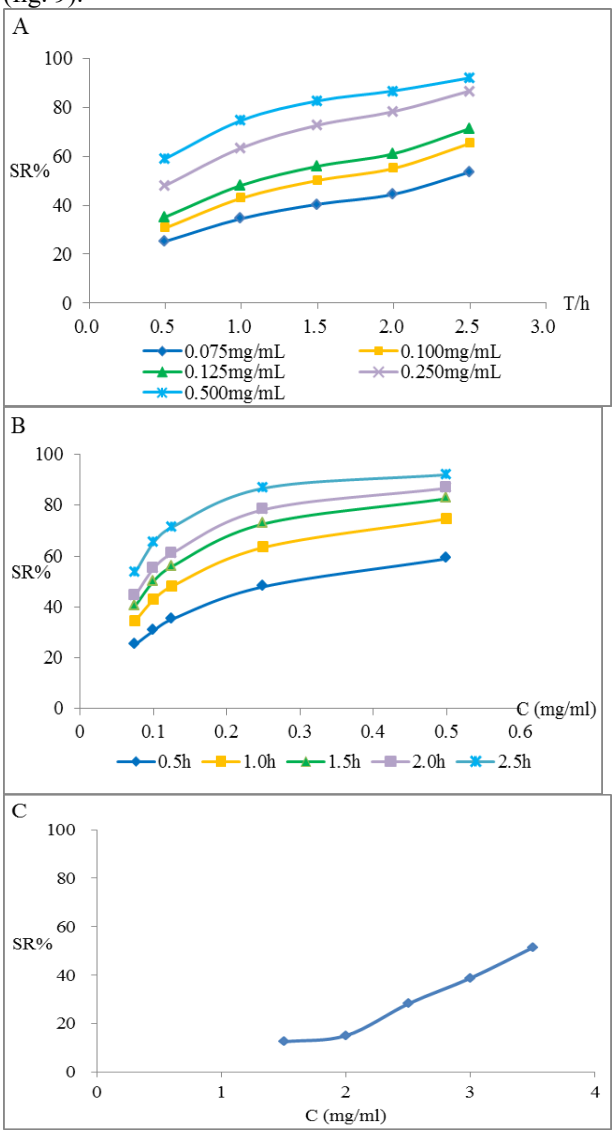


Fig. 6: LTH and LTH-L reacted with DPPH solution. (A) The color development mechanism of DPPH (B) 0 is blank group, 1 to 5 are LTH solutions with low to high concentration (C) 0 is blank group, two different concentrations of LTH-L and the corresponding of BL.

Inhibitory effect of LTH and LTH-L on MDA production from LDL

According to the data, the higher the concentration of LTH, the greater the IR% was. This indicates that LTH had antioxidant activity and could inhibit the oxidation of LDL into MDA. MDA would react with TBA to develop color (fig. 8), when the color reaction with TBA was not obvious, MDA was hardly detected. It can be concluded that the higher the concentration of LTH was, the better the antioxidant activity was and the more it could inhibit the oxidation of LDL. The IR% of LTH and LTH-L (0.0125mg/mL) were 30.82±0.93% and 1.7±0.01%. The IR% of LTH on MDA was almost 18 times that of LTH-L, the difference was more statistically significant (P<0.001) (fig. 9).



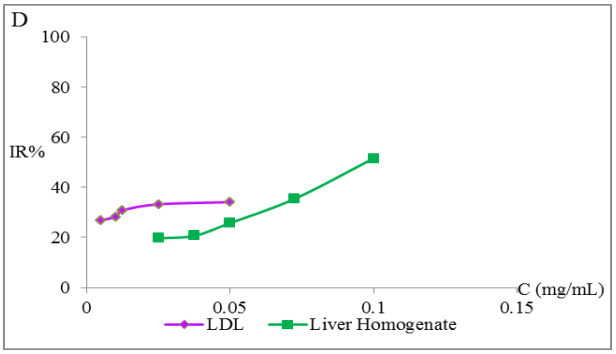


Fig. 7: Anti-oxidation curve of LTH. (A) The SR% on DPPH• by LTH with the same concentration at the different time each curve (B) The SR% on DPPH• by different concentrations of LTH at the same time each curve (C) The SR% on H_2O_2 by LTH with different concentration (D) The IR% on MDA in liver homogenate and LDL by different concentrations of LTH.

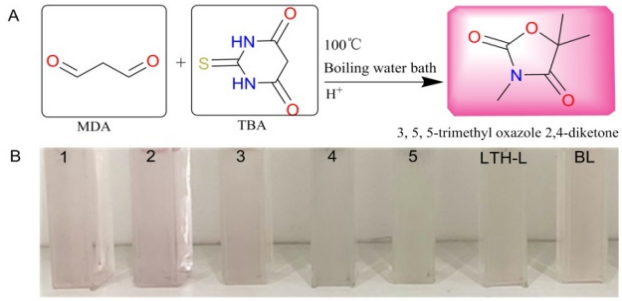


Fig. 8: LTH reacts with LDL. (A) Color reaction of MDA with TBA. (B) Result of color reaction between different concentration of LTH and LDL.

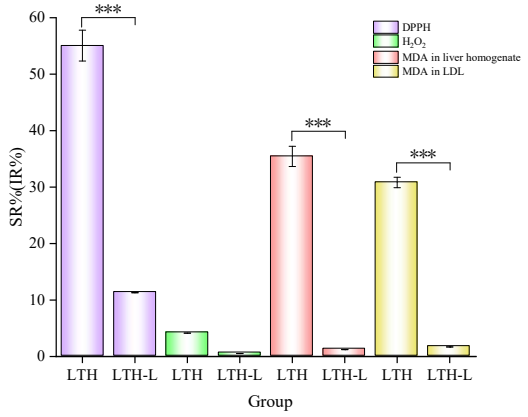


Fig. 9: The SR% and IR% on DPPH•, MDA in liver homogenate and MDA in LDL by LTH and LTH-L. *** P<0.001, which obtained by SPSS Software 26.0 (IBM, Armonk, NY, USA) by an analysis of variance (ANOVA) showed that the difference was very significant.

Study on the antioxidant activity of phospholipid, which is the main component of BL

For further verification, we also did the same experiments on BL that were scavenged DPPH· and H_2O_2, inhibited MDA in liver homogenate and in LDL, BL was diluted by the same multiple as LTH-L in the above experiment. It was found that the SR% on DPPH• of BL was 37.25±1.12%. At the same time, the SR% of BL on H_2O_2 was 75.15±6.10%, it also showed that BL played a great role in anti-oxidation. And the results showed that BL also inhibited the production of MDA, the IR% of BL on MDA production in liver homogenate was 17.92±0.29% and the IR% of BL on MDA production in LDL was 25.19±0.60% (fig. 10).

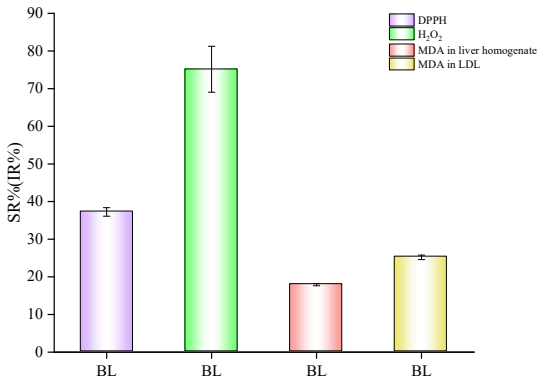


Fig. 10: The SR% and IR% by BL on DPPH•, MDA in liver homogenate and MDA in LDL.

DISCUSSION

Results of particle size, zeta potential and EE% of LTH-L

The efficacy of liposomes was closely related to their physical properties. Measured and analysed the parameters of liposomes, such as particle size and zeta potential were helpful to predict the therapeutic effect of the preparation in a clinical environment.

There was almost no change in particle size, zeta potential and EE% of liposomes after two weeks of storage, which indicated that the pharmaceutical properties of liposomes were basically stable. EE% was an important index of liposomes as quality control. There were many factors that affect EE%, such as the pH value of PBS, the amount of cholesterol and so on. In addition, The EE% of liposomes prepared by different preparation methods would be different; the EE% of LTH-L prepared by thin film dispersion method was relatively high.

Release rate across dialysis membrane and transdermal experiments of LTH and LTH-L

The permeability of drugs via mouse skin was less than that of dialysis membrane, because there was still a big difference between mouse skin and dialysis membrane. The structure of skin was complex, which includes cuticle, epidermis, dermis and hair follicle, etc. Therefore, the transdermal permeability quantity of LTH-L across the

skin was lower than that across the simple dialysis membrane.

Liposomes are a new drug carrier that similar to cell membrane in structure and composition (Zeng *et al.,* 2017), when LTH-loaded liposomes are administered through the skin, the drug molecules stay between epidermis and dermis, forming a drug reservoir and reduced the number of drug molecules entering blood circulation, thus achieved the effect of sustained and controlled release drugs. In the field of skin treatment, external preparations such as emulsion and ointment are not easy to penetrate through the natural barrier of skin and the absorption effect is not ideal, thus failed to achieve a good therapeutic effect, while liposomes can overcome these shortcomings well. Liposomes are a new kind of preparation with nano-structure formed by the lipid bilayers (Liu, Wei, Ye, Tian, & Han, 2017). Lipid bilayer mainly consists of phospholipids and cholesterol (M. Li *et al.,* 2019; Liu, Ye, Han & Han, 2019). Phospholipids are biologically active substance with certain nutritional value. For example, it can reduce blood lipids and delay aging. Its structure is divided into hydrophilic head and lipophilic tail of phospholipids (fig. 11) (Urooj Afreen1 & Shahzad, 2021). Compared with the traditional pharmaceutical dosage forms, liposome has better target and fewer adverse reactions; it is an excellent carrier for drug delivery to and through the skin. Liposomes enter the body via the help of the phagocytosis of cells (Li, Tatematsu, Somiya, Iijima & Kuroda, 2018). When the liposomes pass across the bio-membrane of body and local membranes of phagocytic cells are invaginated, thus engulfing drug molecules and extracellular fluid into the vesicles of the intracellular membrane, these vesicles will enter the biological target site and finally achieve the therapeutic effect (Jinkai *et al*., 2021).

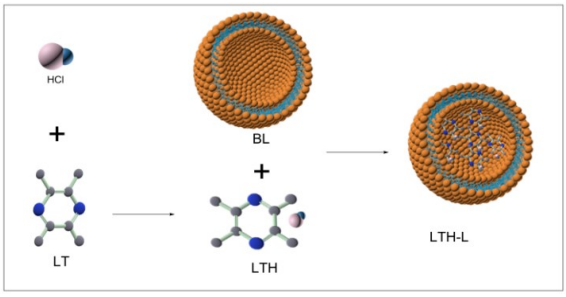


Fig. 11: The process of liposome encapsulating LTH to form LTH-L.

The results of scavenging DPPH· and H_2O_2

DPPH• (Ionita, 2021) has a single electron on the N atom that can form p-π conjugation with the benzene ring. It is the main reason that keeps it stable. DPPH• is dark purple in the methanol or ethanol solution and the maximum absorption peak is at 517nm (Massounga Bora, Li, Liu & Zhang, 2021). When antioxidant and other free radical scavenger are added to DPPH solution, single electrons pair and deep purple DPPH• is reduced to yellow DPPH-H molecule, Its fading degree is quantitatively related to the number of accepted electrons, so it can be quantified by the change of absorbance. When DPPH• reacts with antioxidant, the absorption value at the wavelength of 517nm decreases, which reflects the activity level of antioxidant in scavenging free radical. That is, the stronger the antioxidant activity is, the more obvious the fading degree is, which can be determined by spectrophotometer.

The human body can metabolize and produce various reactive oxygen species, including hydrogen peroxide. In the transmission of electron through the respiratory chain in human body, they will be transferred to oxygen, thus generating H_2O_2 (E *et al*., 2021). Under normal conditions, there is catalase in the human body, which can quickly decompose H_2O_2 and prevent it from damaging cells. However, when the body is under pathological conditions, H_2O_2 is difficult to be decomposed and eliminated, thus causing toxic effects on cells, such as damage to endothelial, cardiovascular and cerebrovascular cells. At this time, we can use antioxidants to resist H_2O_2, thus reducing the oxidative damage of cells.

LTH encapsulated in liposomes had a small scavenging efficiency on DPPH• and H_2O_2, which also indicated that the EE% of liposomes was good, most of LTH was encapsulated into the liposomes structure and would not be released in a short time obviously, at this time, it was the un-encapsulated part that played the role in scavenging DPPH• and H_2O_2. However, the unencapsulated drugs were only a small part, so the SR% of this part should be much lower than that of LTH. If the reaction time was longer, maybe LTH-L could play a good therapeutic role when the body produced excessive oxygen and damaged cells due to oxidative stress.

Inhibitory effect of LTH and LHT-L on MDA that production in liver and from LDL

MDA is a signal molecule of oxidative stress response of organisms. When oxidative stress occurs, a lot of reactive oxygen species and oxygen free radicals are produced. Active oxygen and oxygen free radicals can oxidize phospholipids (Tsikas, 2016) and the oxidized phospholipids undergo a series of enzymatic or non-enzymatic reactions to produce MDA, which will have a strong toxic effects once it is accumulated *In vivo* (Ahilanandan & Anthony, 2021; Barrera *et al*., 2016).

The low density lipoprotein (LDL) is a kind of low density plasma lipoprotein, which contains about 25% protein and 49% cholesterol and cholesterol esters and can bring cholesterol into peripheral tissue cells. LDL can be oxidized into oxidized LDL by oxidants and free radicals (A *et al*., 2019; RuiXia *et al*., 2020), for example, LDL extracted would be oxidized to produce MDA in the

presence of oxidant *ex Vivo* (Moriyama & Takahashi, 2017). MDA can cause cross-linking polymerization of biomacromolecules such as protein and nucleic acid, damage nervous system and affect the function of mitochondria (Lixia *et al*., 2021), it is very important to inhibit the production of MDA.

Study on the antioxidant activity of phospholipid, which is the main component of BL

The liposomes were prepared from phospholipid and cholesterol, both of which had an antioxidant effect. They became oxidized phospholipid and cholesterol after exerting antioxidant effect. Although the BL was not contained LTH, it contained soybean phospholipids and cholesterol, so it could play a role in anti-oxidation. Therefore, we can draw a conclusion that the antioxidant effect of LTH-L was double when LTH was encapsulated in liposomes, not only the antioxidant effect of LTH, but also the antioxidant effect of soybean phospholipid and cholesterol. The liposomes prepared with soybean phospholipids, cholesterol and LTH could exert better antioxidant function, which was the synergistic effect.

CONCLUSION

LTH has good antioxidant properties, dilates blood vessels and resists platelet aggregation, commonly used in cardiovascular and cerebrovascular diseases such as ischemic stroke. Antioxidant activity of LTH plays a great role in the oxidative damage of cells, which can protect cells from oxidative damage or reduce the degree of oxidative damage. Cells contain antioxidant enzymes such as superoxide dismutase and glutathione. When oxidative stress occurs, LTH can increase the synthesis and activity of these enzymes. Moreover, some studies have shown that LTH also has the function of directly reducing the generation of free radicals, which greatly increases the function of scavenging oxygen free radicals in the body and reduces the degree of oxidative damage. Antioxidant experiments *in vitro* and *ex vivo* showed that LTH-L could exert better antioxidant activity, inhibit the production of MDA and further improve its bioavailability. LTH encapsulated in liposomes may not play an obvious antioxidant role in a short time, but the advantage of choosing phospholipid to prepare liposome was that phospholipid itself had antioxidant property, although its antioxidant effect was weak. When combined with other antioxidants, such as LTH, they play a synergistic role, the antioxidant effect of the two together is far greater than that of LTH itself and the antioxidant effect is more obvious. Therefore, it was a good choice to encapsulate LTH in liposomes. Applying LTH-L onto the skin maybe not only prevent diseases in the medical field, but also have whitening effect in the field of cosmetics, because the characteristics of liposome can help LTH enter the skin.

ACKNOWLEDGMENTS

This project was financially supported by Anhui University Natural Science Research Project of Department of Education Anhui Province of China (Grant number: KJ2019A0470, KJ2020A0222, KJ2019A0943), Natural Science Foundation of Anhui Province of China (Grant number: 1608085MH227), Anhui University of Chinese Medicine annual innovation training program for College Students (Grant number: 2017142, 2017171) and Quality Engineering Project of Anhui University of Chinese Medicine (Grant number: 2021zlgc042) and Anhui Provincial Department of Education in 2021 (2021jyxm0824).

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