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Fabrication of *Ligusticum chuanxiong* polylactic acid microspheres: A promising way to enhance the hepatoprotective effect on bioactive ingredients

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Abstract

Ligusticum chuanxiong extract-polylactic acid sustained-release microspheres (LCE-PLA) are fabricated in this study for enhancing both duration and hepatoprotective efficacy of the main bioactive ingredients. LCE-PLA *in vitro* release, cytotoxicity and *in vivo* hepatoprotective effect were discussed to evaluate its efficiency and functionality. Results demonstrated that the optimal drug-loading rate and encapsulation efficiency of tetramethylpyrazine (TMP, the main active ingredient) were 8.19%, 83.72%, respectively. The LCE-PLA *in vitro* release of TMP showed prolong 5-fold and *in vitro* cytotoxicity declined 25.00% compared with naked LCE. After 6 weeks of *in vivo* intervention in high fat diet mice, both liver aspartate aminotransferase and alanine aminotransferase levels were higher in LCE-PLA group than LCE group. The above results indicated that TMP had a higher bioavailability of hepatoprotection when encapsulation of LCE-PLA was applied. The current study has provided a promising novel way to enhance the efficacy of short half-life ingredients.

Keywords: *Ligusticum chuanxiong*, sustained-release, microspheres, high fat diet, hepatoprotective effect

1 Introduction

Long-term intake of high fat food (HFD) increases the body weight and results in obesity, and in turn causes serious damage to the liver like fatty liver and cirrhosis even hepatocellular carcinoma (Qin, Zhao, Zhou, Zhang, Wen, Tang, et al., 2018). Dietary therapy has been recommended for treatment of liver disease, especially for the cirrhosis and nonalcoholic fatty liver disease (Mager, Iniguez, Gilmour, & Yap, 2015). Natural herbal dietary supplements are the most popular pathway due to its complex mechanisms as multi-compound, multitarget and multi-pathway (Brodziak-Dopierala, Fischer, Szczelina, & Stojko, 2018; Wang, Li, Zhao, Liu, Liu, Yang, et al., 2013). Traditional hepatic-protecting herbal plants, such as *Ligusticum chuanxiong*, *Salvia*, *Angelica*, *Panax notoginseng*, *Astragalus*, etc., have been reported to reduce serum aminotransferase degeneration and necrosis of liver cells and show anti-fibrosis effect (Gao, 2013).

Ligustrazine, also known as tetramethylpyrazine (TMP), is the major alkaloid in *Ligusticum chuanxiong*. TMP, with a simple structure, is well studied because of its multiple significant biological functions, such as inhibiting apoptosis, dilating blood vessels, protecting vascular endothelial cells and immune regulation, eliminating oxygen free radical, improving cerebral ischemia, inhibiting platelet aggregation, and promoting angiogenesis, effectively attenuate liver injury etc. (Mo, Liu, Li, Xu, Wen, Xian, et al., 2017; Yu, Guo, Zhang, Liu, Zou, Fu, et al., 2016). TMP has been extensively used for developing health-promoting foods.

However, the shortcoming of fast metabolism, short half-life (1.62 h), and poor

oral availability limit TMP application in liver therapy (Li, Song, Zhu, Yin, Ji, Li, et al., 2014). Sustained-release microspheres have been reported to help prolong the acting time of medicament, improve patient compliance and reduce side effects (Wu, Hu, & Jin, 2016). In addition, the majority studies on the sustained-release microspheres are based on the *in vitro* experiments such as antioxidant and cytoactive, but rare evaluation on the *in vivo* function and cytotoxicity. In this study, *Ligusticum chuanxiong* extract (LCE) was used to make sustained-release microspheres to enhance its bioavailability and efficiency of hepatoprotective.

Polylactic acid (PLA), as a biodegradable polymer with the characteristic of high elastic modulus, high mechanical strength, and feasible processability, has been widely used in the preparation of slow-controlled release microspheres, especially in the preparation of composite microspheres (Surwase, Munot, Idage, & Idage, 2017). Traditional emulsion solvent evaporation method has been widely used to encapsulate hydrophilic drugs in polymeric particles (Murphy & Lampe, 2018). LCE can be soluble in water, W/O/W (water-in-oil-in-water) multiple emulsion technique is a useful method for water-soluble drug encapsulation (Bodmeier, Wang, & Bhagwatwar, 1992).

In the present study, LCE was incorporated in W/O/W multiple emulsions with PLA as the carrier material, tween-80 and PVA as the co-emulsifier. The technologies for encapsulating LCE were optimized and the microspheres were characterized with the drug-loading rate (DL), encapsulation efficiency (EE), morphology and *in vitro* cytotoxicity. Swelling ratios of the microspheres at different pH was evaluated for

unveiling the mechanism of the *in vitro* release profiles. Furthermore, the hepatoprotective effects in mice were evaluated. The outcome will favor the application of *Ligusticum chuanxiong* on liver therapy.

2 Materials and methods

2.1 Materials

Ligusticum chuanxiong (origin: Sichuan, the production license: 20160084). TMP standard (purity≥99.98%) was purchased from Shanghai Yuan ye Biotechnology Co. Ltd. Polyvinyl alcohol 124 (PVA, 1.05×10^5 Da). Chemical pure Tween-80, analytical reagent dichloromethane (DCM), analytical reagent methanol, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 7H_2O$ and potassium bromide were purchased from Sinopharm Chemical Reagent Co., Ltd. Polylactic acid powder (4500 Da) was purchased from America Nature Works. The defoaming agent (WH-Z) was purchased from Changzhou Jiangdong auxiliaries Co., Ltd.; Total triacylglycerols (TG), Cholesterol (TC), Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) kits were purchased from Nanjing Jiancheng Biological Engineering Institute.

2.2 Preparation of *Ligusticum chuanxiong* extraction

Using 95% ethanol to reflux extraction 100.00 g *Ligusticum chuanxiong* powders (1:8, w/v) for 2 times and then freezing dried the extraction liquid for further use.

2.3 Preparation of LCE-PLA

Ligusticum chuanxiong extract-polylactic acid sustained-release microspheres (LCE-PLA) were prepared through the emulsion solvent evaporation technique (Petkar, Chavhan, Kunda, Saleem, Somavarapu, Taylor, et al., 2018). LCE was

dissolved in distilled water (10 mg/mL) at 40 °C to prepare the internal water phase (W_1). The oil phase (O) was prepared by mixing PLA with DCM (PLA/DCM, w/v) and stirring for 30 min at 24 °C. The external aqueous phase (W_2) was prepared by adding PVA and Tween-80 (co-emulsifier) and shearing with a homogenizer at 9000 rpm for 2 minutes.

Phase W_1 was added dropwise into the organic phase (O) to make W_1 /O primary emulsion. W_1 /O/ W_2 composite emulsion was herein obtained by adding phase W_2 into the primary emulsion. The composite emulsion was homogenized to prepare stable multiple emulsions. After that, the organic phase was vaporized at 40 °C to get solid microspheres. The produced microspheres were collected by centrifugation at 6,000 rpm, washed thrice with distilled water, and lyophilized overnight (Surwase, Munot, Idage, & Idage, 2017).

2.4 Determination of drug-loading rate and entrapment efficiency

TMP in LCE were quantified by high performance liquid chromatography (HPLC) according to our previous method (Ge, Chen, Chen, Tian, Liang, & Chen, 2018). In brief, Waters XBridge C18 reversed phase column (600 Bar, 250mm×4.6 mm, 5 μm) was used for chromatographic analysis. The mobile phase is methanol: 1% glacial acetic acid aqueous solution= 45:55; velocity of flow is 0.80 mL/min; detection wavelength: 310 nm. Content of TMP in LCE is 55.69 mg/g.

The DL and EE of TMP in microsphere were determined by the above HPLC method after the following pretreatment steps. Firstly, ten mg of microspheres was suspended in 10 mL of methanol and subjected to ultrasonic treatment at 80 W for 10

min and centrifuged the mixture at 10000 rpm for 10 min. Secondly, taken 1 mL supernatant were diluted to 10 mL with methanol and filtered through 0.22 mm membrane before determination. The DL and EE were calculated by following equations (Duan, Zhang, Chu, Tong, Liu, & Zhai, 2016):

$$DL = Weight\ of\ TMP\ in\ microspheres / Mass\ of\ microsphere \times 100\% \quad (1)$$

$$EE = Drug\ encapsulated\ in\ microspheres / Weight\ of\ drug\ added \times 100\% \quad (2)$$

2.5 Optimization preparation of LCE-PLA

PLA concentrations (20, 30, 40, 50 and 60 mg/mL), PVA contents (0.60%, 0.80%, 1.00%, 1.20% and 1.40%), shearing times (2, 3, 4, 5 and 6 min) and shearing speeds (7000, 8000, 9000, 10000 and 11000 rpm) were set as the factors to investigate the DL and EE of TMP in composite microspheres. During the preparation, Tween-80 and PVA was used as a co-emulsifier, the scale of W_1 and W_2 was 1:20. The evaporation rate of solvent was set at 300 rpm for 4 h.

2.6 Fourier transform infrared spectroscopy analysis

Fourier transform infrared (FT-IR) spectra of pure PLA, LCE and LCE-PLA obtained on a WQF-510 spectrometer (Campardelli, Oleandro, & Reverchon, 2016). Each sample was prepared in KBr discs (1.00%, w/w), and the scans were conducted over the range from 4000-400 cm^{-1}. For each spectrum, 64 scans with air as the background were obtained at a resolution of 4 cm^{-1}. Spectra over the range of 4000-400 cm^{-1} were baseline corrected automatically, normalized and deconvoluted.

2.7 Morphology and particle size of microspheres

The morphology of LCE-PLA was observed by scanning electron microscopy

(SEM) (Petkar, et al., 2018). Samples were mounted onto the metal holder and gold coated in a vacuum chamber, then recorded images at 15 KV acceleration voltage by JSM-6380LV SEM (JSM-6380LV, JEOL, Tokyo, Japan).

The mean particle size of the W/O/W microsphere was measured by a laser particle-size analyzer (Beckman, Brea, CA, USA). The dry microspheres were placed in deionized water and stirred at 3000 rpm and sonicated at 50 mV until the light-blocking ratio between 5%-13%. Each sample measure three times. Afterwards, analyses the particle size of the W/O/W microspheres by the software provided. The median of particle size (D_{50}±SD) was reported as the particle size of microspheres (Bagheri-Khoulenjani, Etrati-Khosroshahi, & Mirzadeh, 2010).

2.8 *In-vitro* drug release and swelling ratio

LCE-PLA *in-vitro* release behaviors were determined according to the method of Surwase, Munot, Idage and Idage (2017). Fifty mg of microspheres was suspended in different phosphate buffer solutions (pH 1.2, pH 6.8, pH 7.4) to make the microspheres solution, then put the solutions into 3 dialysis bags (MW, 14000 Da) (Lin, Huo, Qin, Zhao, & Tao, 2017). Afterwards, the dialysis bags were put into 28 mL phosphate buffer (pH 1.2, pH 6.8, pH 7.4) and maintained at 37±1 °C with 100 rpm agitating for 10 hours. Withdraw 3 mL aliquots every half an hour, and an equal volume was replaced with fresh phosphate buffer each time and then determined at 279 nm. Finally, quantify the released of drugs, and demonstrated the cumulative release profile with time at different pH conditions. All measurements were performed in three replicates, and results expressed as mean value ± SD.

171 Swelling capacity of LCE-PLA at different pH (pH 1.2, pH 6.8, pH 7.4) were
172 measured based on the method of Khattab and Zaki (2017). Three portions of
173 lyophilized microspheres (each portion of 100 mg) were placed in pre-weighed dense
174 gauze bag and weighed (W_0), then put the bags into different mediums. Taken out the
175 bags every 0.5 h, filter paper was then used to absorb the water attached to the
176 microsphere surfaces, then weighted to get the wet microspheres weight (W_t) until the
177 mass of microspheres keeps constant. Each pH situation was performed in three
178 replicates. The swelling ratio (SR) was calculated as following equation:

179 $$SR = (W_t - W_0)/W_0 \times 100\% \quad (3)$$

180 **2.9 Cytotoxicity assay**

181 To ensure the safety of the LCE-PLA, the cytotoxicity was measured following
182 the method by Chen et al. (Chen, Tian, Ge, Liu, & Xiao, 2017; Yoon & Liu, 2007).
183 Density of 4×10^4/well HepG2 cells and 100 μL growth medium were plated into a
184 96-well plat and incubated at 37 °C for 24 h. After removing the growth medium, the
185 cells were treated with 100 μL fresh medium (WME supplemented with 2 mM
186 L-glutamine and 10 mM Hepes) with different concentrations of TMP, LCE, and
187 LCE-PLA as samples or only Dimethyl sulfoxide (DMSO) as control for another 24 h
188 at 37 °C. After that, each well was added 50 μL methylene blue solution, which
189 contained by 98.00% HBSS, 0.67% glutaraldehyde, 0.60% methylene blue, the plat
190 was stored at 37 °C for 1 h. Using water to rinse the cells until the water was clear and
191 then dried cells. The elution solution, consisting of 49.00% PBS, 50.00% ethanol and
192 1.00% acetic acid, were used to elute the methylene blue stain for 1 h at room

temperature. Finally, the absorbance was read at 570 nm with blank subtraction by an MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). Concentrations of the LCE-PLA or LCE or pure TMP that decreased the absorbance by more than 10.00% were considered cytotoxic compared with the control group. In the present study, the contents of TMP in LCE and LCE-PLA were equal to the pure TMP group.

TMP stock solutions which concentration below 0.5 mg/ml were directly prepared by fresh medium, the concentration exceeded 0.5 mg/ml were dissolved in fresh medium contain 1% DMSO.

2.10 Animals and experimental design

Forty male Kunming mice weighing 20.00 ± 2 g were purchased from the Beijing HFK Bioscience CO..LTD (SCXK 20140004). The mice were housed in Animal Center of Fuzhou General Hospital of Nanjing Military Command at a constant temperature of 25 °C and humidity range of 50-60% with a 12-h light/dark cycle. After acclimatization for one week, the mice were randomly divided into two groups. The first group (normal-fat diet, NFD) is the normal, healthy control group (8 mice) and the second group (Group II) is the HFD group (32 mice). During the 6-week experimental period, all mice in group II were fed with HFD (containing 5.00% sucrose, 10.00% lard oil, 2.00% cholesterol, 0.50% bile salts, 0.20% propyl thiouracil, 5.00% whole yolk powder, 5.00% bentonite and 72.30% NFD). Both NFD and HFD were provided by Beijing Keao Xieli Feed Co. Ltd.

Mice in group II were further classified into four groups: group II-a (HFD),

group II-b (HFD+S, commercial *Simvastatin* treated group at a dose of 6.00 mg/kg B.w./Day), group II-c (HFD+LCE, *Liguticum chuanxiong* alcohol extract treated group at a dose of 136.50 mg/kg B.w./Day) and group II-d (HFD+DM, drug-loading microspheres treated group at a dose of 163.00 mg/kg B.w./Day). In the current study, the LCE feeding dose was calculated according to the daily consumption (10.00 g/day) of *Liguticum chuanxiong* crude rhizome recommended by Chinese medicine, and the extraction yield of LCE is about 9.10%. The dose of TMP in microspheres (EE of TMP 83.72%) was equal to which in LCE. Simvastatin as a positive common drug for treat hyperlipidemia, its dose was according to the clinic recommended by the instructions (Guo, Pan, Li, Li, Liu, & Lv, 2018).

At the end of the experiment, all mice were injected intraperitoneally with 40 mg/kg pentobarbital sodium, then collected blood samples from the abdominal aorta and centrifuged at 3000 g for 10 min to separate serum and stored at -20 °C. After that, all mice were sacrificed by cervical dislocation and dissected. The livers were quickly removed and washed with physiological saline and blotted dry on filter paper, partial liver tissues were taken for hepatic histological analysis by Hematoxylin-Eosin Stain (HE) (Guo, Pan, Li, Li, Liu, & Lv, 2018).

2.11 Statistical analysis

One-way analysis of variance (ANOVA) was performed with Tukey's HSD test ($p<0.05$) using SPSS (17.0 version, IBM). All results were expressed as mean ± standard deviation. The significance level was set as $p<0.05$.

3 Results and discussion

3.1 Preparation optimization of LCE-PLA

Research had found that different preparation process could affect the encapsulation rate of active ingredients (Ebrahimi, Saffari, & Langrish, 2017; Qiu, Zheng, Fang, Wang, Min, Shen, et al., 2018). As indicated in **Table S1**, with the increased of PVA addition level (PVA/Water, w/v), both DL and EE of TMP reached the maximum value at 6.83% and 69.91%, respectively. When the addition of PVA was greater than 0.80%, results of DL and EE showed no significant difference (p>0.05). Therefore, the addition of PVA was selected by 0.80% (PVA/Water, w/v). When the concentration of PLA increased to 40 mg/mL, effect of PLA addition on the DL and EE showed no significant difference (p>0.05). To save energy, the concentration of PLA was selected 20 mg/mL. Effect of shearing time on the DL and EE were significant difference in **Table S1** (p<0.05). During the first 4 minutes, both DL and EE increased with the increase of shearing time. Interestingly, after 4 minutes, with the prolonger of shearing time, results of the DL and EE showed no significant difference (p>0.05). Influences of shearing force on the DL and EE were significant differences (p<0.05) in **Table S1**. With the increase of shearing force, the emulsion sizes become smaller, and the contact area is increased, thus, the DL and EE are increased. When the shearing force more than 1000, there was no significant different (p>0.05), thus chosen the shearing fore at 10000 rpm. To sum up, the reasonable preparation process is the PLA concentration of 20 mg/mL (PLA/DCM, w/v), PVA 0.80% (PVA/Water, w/v), and shearing force at 10000 rpm for 4 min.

3.2 FT-IR spectroscopy

FT-IR spectroscopy was carried out to characterize the interaction mechanism of LCE loaded in PLA microspheres. As illustrated in **Fig. 1**, the characteristic peaks around 3524 cm^{-1} and 3251 cm^{-1} (hydrogen bonds from -OH), as well as 1421 cm^{-1} (stretching vibration of C=O), 1665 cm^{-1} (stretching vibrations of -C=N) and 992 cm^{-1} (C=C) in LCE, disappeared in the LCE-PLA microspheres attributing to the adequate encapsulation process (Zou, Fu, Chen, Austarheim, Inngjerdingen, Huang, et al., 2017). The characteristic peaks at 1391 cm^{-1} (stretching vibration of $-CH_3$), 1756 cm^{-1} (stretching vibration of C=O) and 1095 cm^{-1} (C-O-C) in PLA (Garlotta, 2001; Yuniarto, Purwanto, Purwanto, Welt, Purwadaria, & Sunarti, 2016) were sharply attenuated in the LCE-PLA microspheres might due to the interaction with LCE. Interestingly, the typical band of PLA at 2369 cm^{-1} (stretching vibration of C=O) was also disappeared in the LCE-PLA microspheres resulting from the interaction with LCE, which confirmed the internalization of C=O in PLA during the formation of microspheres.

3.3 Surface morphology of microspheres

Mlalila, Swai, Kalombo and Hilonga (2014) found that higher emulsifier concentrations would decrease the interfacial tension via facilitating particle disruption. When PVA concentrations increased, the surfactants may form tighter micelles around microspheres, and resulted in the small size (Xu, Zhong, Liu, Xu, & Gao, 2011). In **Fig. 2 C**, the particle sizes of microspheres were surely smaller than that of **A**. In the **Table S1**, with the concentration of PVA (PVA/water, v/v) increased, the particle size (D_{50}) declined from 18.78 μm to 10.50 μm, those results were in

281 agreement with the result of Li et al. (2013). When the PVA concentration increased,
282 the surfactants might closely to form tighter micelles around the microspheres, which
283 result in a smaller particle size. However, with the concentration of PLA increased,
284 the mean particle sizes of microspheres increased from 11.41 μm to 17.90 μm in
285 Table1, which can also be found in **Fig. 2 B**.

286 The resultant product by high-shear shows a smaller particle size compared with
287 the emulsion produced by the conventional agitation (Patrick & James, 1997).
288 Different speed resulted in the different particle size of microspheres, with the
289 increasing of shearing speed, the particle size became smaller in **Fig. 2 D**. When
290 shearing time increased, microspheres sizes were also declined in **Fig. 2 E**.
291 Bagheri-Khoulenjani, Etrati-Khosroshahi and Mirzadeh (2010) found that high speed
292 could help to decline the size of microspheres.

293 SEM imaging of empty microspheres and broken microspheres were also studied
294 (**Fig. S1 A**, **C**). The broken microspheres were prepared by a high temperature and
295 high-pressure reactor (HTLAB). LCE-PLA was basically round, but slightly
296 deformed when compared with the empty microspheres, this might be the cause of
297 LCE combined into microspheres added the weight of microspheres. The broken
298 microspheres SEM photographs were used to imitate the state of the microspheres
299 after fully swelled and ruptured in the body (Chu, Liu, & Chen, 2018).

300 Both Tween-80 and PVA is commonly used as emulsifier in preparation
301 microspheres. The research found that the co-emulsifier can help improving
302 emulsifying performance (Zhang & Haque, 2015). In the present study, Tween-80 and

PVA is used as the co-emulsifier to help enhance the quality of microspheres. Due to its high viscosity in aqueous solution, strong adsorption around the surfaces of the emulsion droplets, PVA could help make a smooth-surface and spherical morphology of microspheres (Feng & Huang, 2001) like the microspheres in **Fig. 2** and **Fig. S1**.

3.4 Microspheres swelling and ratio *in-vitro* drug release

As illustrated in **Fig. 3 A**, the *SR* of microspheres increased following by time under different medium. At 3 h, the S_{Max} value is 11.82% (pH 1.2), 15.02% (pH 6.8), and 16.19% (pH 7.4), respectively. Microspheres can be swelled well during the weak acidic and weak basic conditions. When the medium pH at 1.2, the *SR* increased slower compared with the other two mediums. Reports had found that process of swelling was related to the ionization and the protonation balance of the carboxyl groups. TMP mainly absorbed in the small intestine, during those conditions, the -COOR of PLA can slowly react with the basic condition solution (Metters, Anseth, & Bowman, 2000). TMP can be released in the intestine condition absolutely and prolonged the half-life.

The accumulative releasing rate of TMP in LCE-PLA under different pH media (pH 1.2, pH 6.8, pH 7.4) were shown in **Fig. 3 B**, the release could be sustained at least 10 h. Comparing with the TMP half-life, the microspheres duration of efficacy prolonged nearly 5-fold. Generally, the hydrolytic mechanism was considered as the mechanism of degradation of aliphatic polyester microspheres (Pistner, Bendix, Mühling, & Reuther, 1993) With the penetration of water, the prepared W/O/W PLA microspheres were substantially bulk erosion, degraded, and followed with the slow

diffusion of encapsulated drug, those results were consistent with the reports by Ford Versypt et al. (Ford Versypt, Pack, & Braatz, 2013; Li, Wang, Yu, Bao, & Li, 2013).The prepared microspheres were first swelling in each condition solution, during this process, because of the ionization and the protonation balance of the carboxyl groups, the carrier material PLA can be absolutely hydrolysis especially in the weak basic conditions and released the activity compounds (Metters, Anseth, & Bowman, 2000). The *SR* of microspheres was reached to the highest at 3 h in **Fig. 3 A** and result in a high-speed release in **Fig. 3 B**. After 2 h, the cumulative releasing rate was reached to 38.00% of the median of pH 1.2 while the rate was over 50.00% at pH 6.8 and pH 7.4. At 6 h, there was no significant difference in the release rate of microspheres between pH 6.8 and pH 7.4 (p>0.05). Result suggested that during the environment of weak acid or a weak base, the microspheres could be dissolved as well, which also indicated that the W/O/W composite microspheres could prolong the time of drug action. Research has found that a single administration of drug-loaded polymer microsphere could help the release of drug in a long period by the way of continuous and controlled, therefore, the drug concentration could retain in the within target (Ma, Yuan, Kang, Su, Yuan, Pu, et al., 2008).

3.5 Result of cytotoxicity and hepatotoxicity

Cytotoxicity as a significant criterion has been widely used to determine the suitability of polymers in drug delivery (Petkar, et al., 2018). In this paper, there were no significant difference in percent cell viability of LCE-PLA compared to the LCE, pure TMP and control group when the drug concentration below 0.8 mg/mL in **Fig. 4**.

The cytotoxicity of pure TMP, LCE and LCE-PLA became evident at the concentration of 1.50 mg/mL, 0.80 mg/mL, and 1.00 mg/mL, respectively. Cytotoxicity of LCE was declined 25.00% compared with LCE. Coowanitwong, Arya, Kulvanich and Hochhaus (2008) found that Rifampin-PLA microsphere formulations were low cytotoxicity.

To investigate the hepatotoxicity of treatment contents, the hepatic TG, hepatic TC AST, ALT and hepatic histopathology of liver and kidney in all groups were determined. As shown in **Fig. 5 A**, the HFD groups mice hepatocytes were markedly swollen and enlarged, and it was easy to find that the hepatic nuclei were compressed to side and there were many fat vacuoles in the cytoplasm. In contrast, simvastatin, LCE and microspheres treatment improved the cellularity of liver cells, and smaller lipid droplets were observed compared with HFD. The liver weight in HFD, HFD+S, HFD+LCE, HFD+DM groups was increased in **Table 1** compared with NFD group. The values of the above group livers weight Δ a (%), Δ b 1 (%), Δ c 1 (%) and Δ d 1 (%) in **Table 1** was 31.34, 10.45, 24.88 and 11.44, respectively. Report had found that the increase of liver coefficient could indicate edema, inflammation, or hyperplasia in the liver tissue (Bezan, Mrsic, Krieger, Stojakovic, Pummer, Zigeuner, et al., 2015). Results indicated that LCE-PLA had a helpful effect on the liver injury mice induced by HFD. And the effect of above treatment activity compounds followed as: Simvastatin > LCE-PLA > LCE. **Fig. 5 B** showed the histological analysis of kidney sections. In the treatment group, the renal tubules were clear and the cells arranged regularly while the HFD group showed the opposite situation such as narrowing of the

renal tubule, cells fusion and glomerulonephritis. In **Table 1**, NFD group and treatment groups had the same kidney weight, those mice kidney were normal.

AST and ALT are well-known liver enzymes produced by both malignant and non-malignant cells and have been widely used in the evaluation of various causes of liver disease such as viral hepatitis and alcohol abuse etc. (Bezan, et al., 2015; Lee, Lee, Byun, Kim, Kwak, & Hong, 2017). In **Fig. 5 C**, the value of hepatic TG and TC in HFD group were higher than which in treatment groups, while the AST and ALT showed the opposite (p<0.01). When the liver injury, the AST and ALT will be released into the blood and reduced the content in liver tissue. In the current study, in each treatment group, levels of both AST and ALT were as follows: NFD>HFD+S>HFD+DM>HFD+LCE. The above results confirmed that the hepatotoxicity of treatment activity compounds was low and TMP in LCE-PLA could produce a better curative effect.

4 Conclusions

In the present study, LCE-PLA were synthesized and characterized. The proposed LCE-PLA EE was more than 80.00%, and the duration prolonged nearly 5-fold compared with the naked LCE. *In vitro* cytotoxicity assay and *in vivo* hepatoprotective effect confirmed that LCE-PLA enhanced the solubility and bioavailability of active compounds, even declined the cytotoxicity of LCE. Overall, these findings indicated that release microspheres can be a useful way to improve the *Ligusticum chuanxiong* clinical use. Furthermore, this study can provide a promising novel way to enhance the efficacy of short half-life ingredients.

Ethics statement

In current study, all animal experimental procedures followed the National Institutes of Health guide for care and use of Laboratory animal (NIH Publications, No.8023, revised 1978), and they were performed in strict accordance with the China legislation regarding the use and care of laboratory animal. The present experiment was approved by the Welfare and Ethics Committee of Fuzhou General Hospital of Nanjing Military Command (No. IACUC-2018-0009).

Declaration of competing interest

The manuscript has been reviewed and approved by all authors. All authors declare no conflict of interest.

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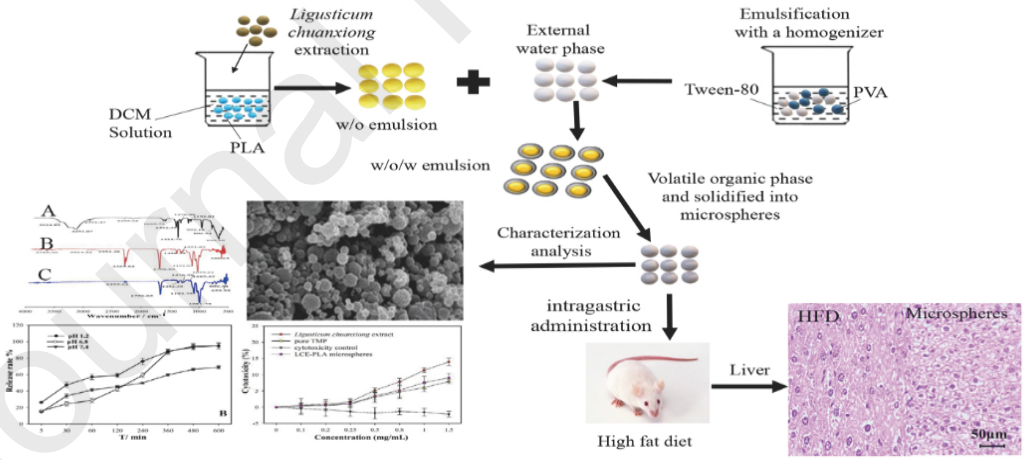

Highlights

- Sustained-release microspheres encapsulation efficiency of TMP are more than 80%
- LCE-PLA microspheres duration of efficacy prolong nearly 5-fold
- LCE-PLA microspheres cytotoxicity declined 25% than naked LCE
- LCE-PLA microspheres had a significant hepatoprotective effect

Credit Author Statement

Huifang Ge: Designed and participated the experiment, Writing-Original draft preparation. Jicheng Chen: Designed the experiment and revised the manuscript and was responsible for the supervision of the whole research. Peixuan Lin: participated in the experiment and revision of the paper. Taiduan Luo: participated in the experiment and analysis the experimental date. Zhiming Yan and Jianbo Xiao had provided guidance for publication, and help revision the paper. Song Miao: Writing- Reviewing and was responsible for the supervision of the whole research.

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Figures Captions

Figure 1 FI-RT of *Ligusticum chuanxiong* extraction (LCE), polylactic acid (PLA) and *Ligusticum chuanxiong* extract-polylactic acid microspheres (LCE-PLA)

Figure 2 SEM of *Ligusticum chuanxiong* extract-polylactic acid microspheres (LCE-PLA) under different preparation technology. PVA, polyvinyl alcohol; DCM, dichloromethane. A to E is the SEM of drug-loading microspheres. The samples were observed using SEM with voltage 15 kV and 10000 x magnification. A is the microspheres made on the situation of PLA 20 mg/mL (PLA/ DCM, m/v), PVA 0.80% (PVA / water, m/v), and shearing for 4 min at 9000 r/min; B changes the PLA to 30 mg/mL (PLA/ DCM, m/v) based on A; C changes the PVA additive amount of 1.00% (PVA / water, m/v) compare to A; D the shearing force is changed to 10000 r/min; E changes the shear time to 6 min based on A.

Figure 3 Swelling curves (**A**) and cumulative release (**B**) of *Ligusticum chuanxiong* polylactic acid microspheres under different pH media (Mean ±SD, n=3)

Figure 4 Effect of *Ligusticum chuanxiong* extraction (LCE), *Ligusticum chuanxiong*-polylactic acid (LCE-PLA) microspheres and pure tetramethylpyrazine (TMP) on cytotoxicity in HepG2 cells (Mean ±SD, n=3). In the present study, the contents of TMP were equal in both LCE and LCE-PLA microspheres.

Figure 5 Representative histological analysis of liver sections (**A**) and kidney sections (**B**) from mice fed on NFD (normal-fat diet), HFD (High fat diet), HFD+S (HFD + 6.00 mg/kg B.w./Day *Simvastatin*), HFD+LCE (HFD +136.50 mg/kg B.w./Day *Liguticum chuanxiong* alcohol extract), HFD+DM (HFD +163.00 mg/kg B.w./Day drug-loading microspheres) and drug-loading microspheres for 6 weeks and hepatic total cholesterol (TC), triglyceride (TG), aspartate aminotransferase (ALT), alanine aminotransferase (AST) levels in each group (**C**). Values are means ± SD (n=8). $^{\#}p<0.05$; $^{\#\#}p<0.01$ means statistically significant difference compared to the NFD group; $^{*}p<0.05$; $^{**}p<0.01$ means statistically significant difference compared to the HFD group.

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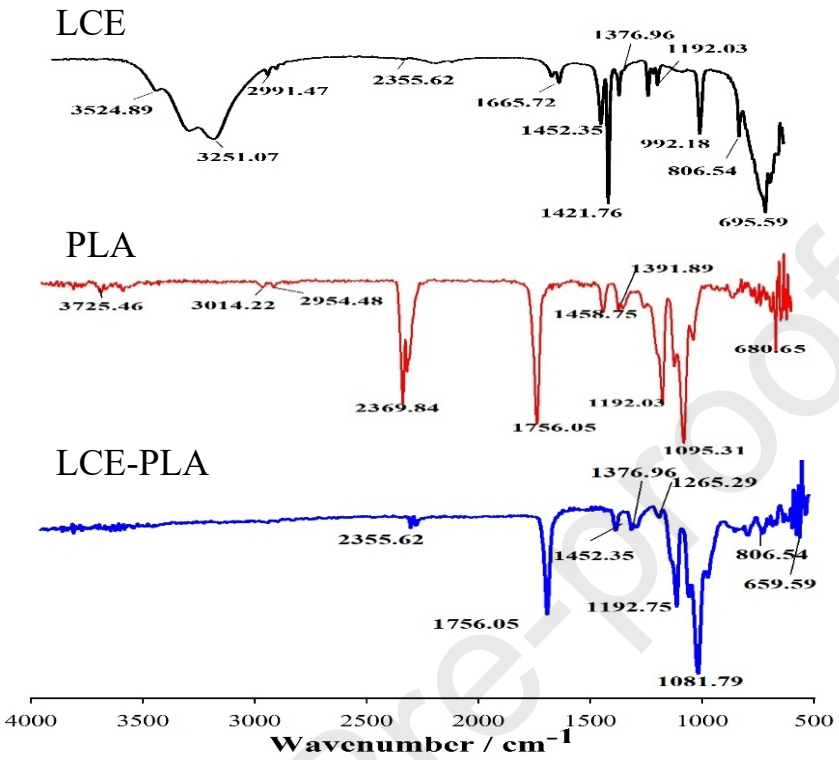
615 **Figure 1**



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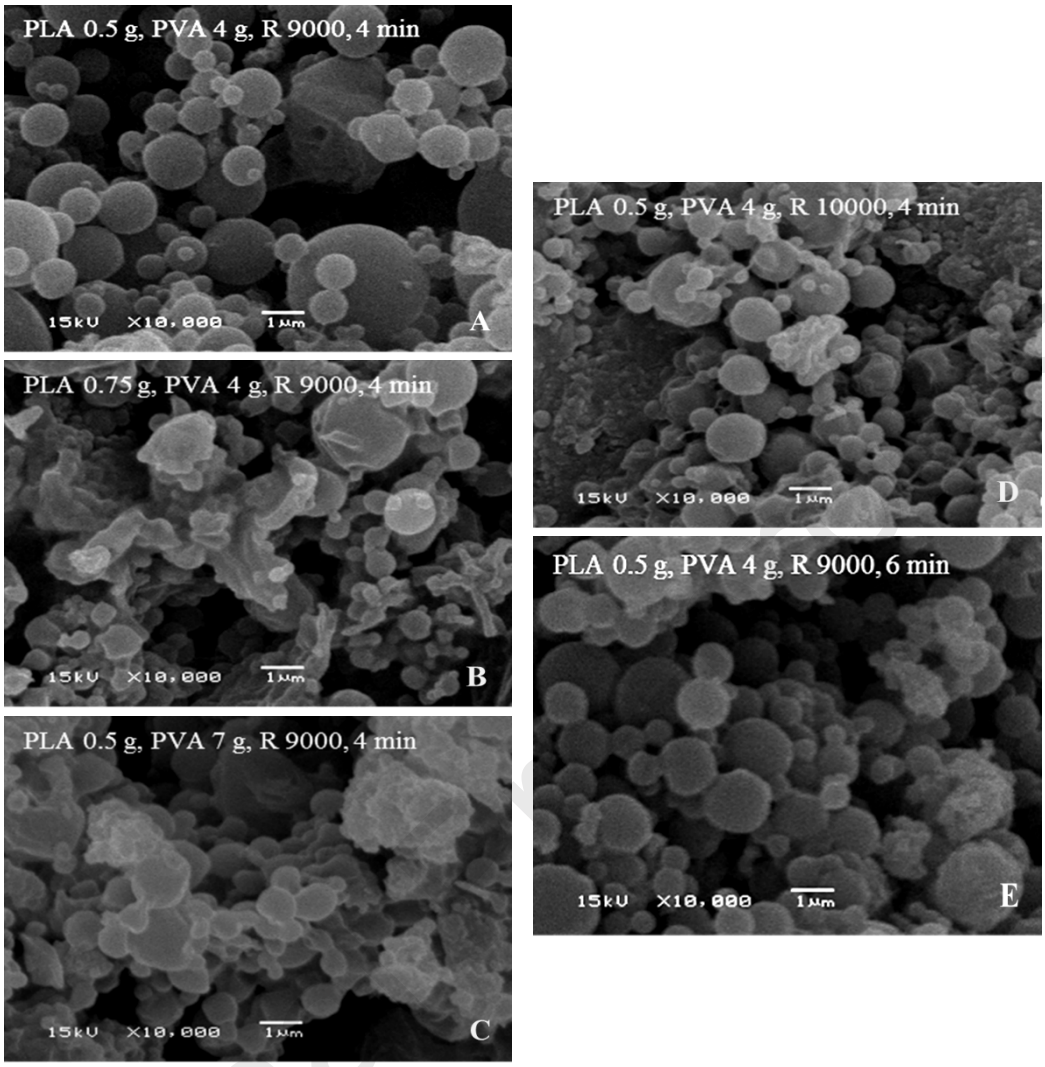
619 **Figure 2**



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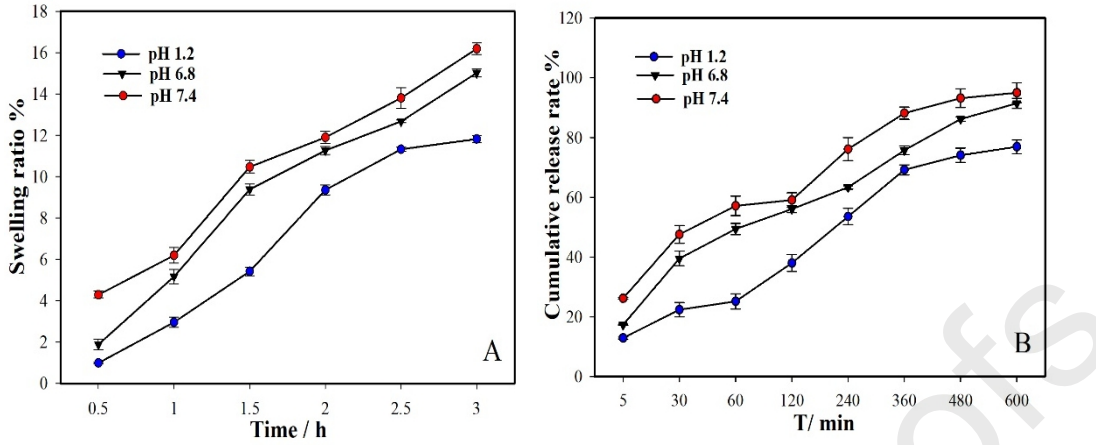

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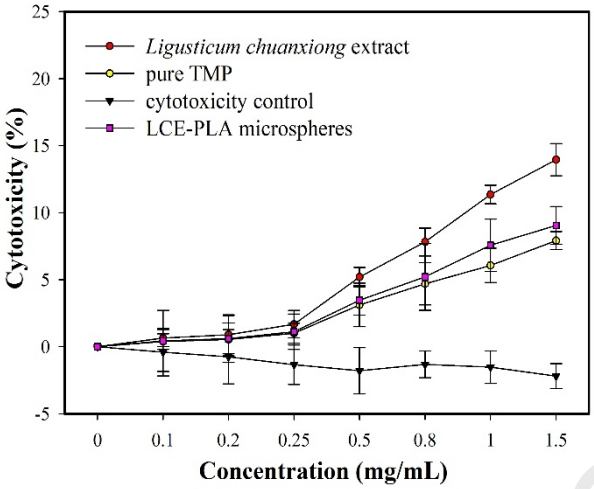
625 **Figure 4**



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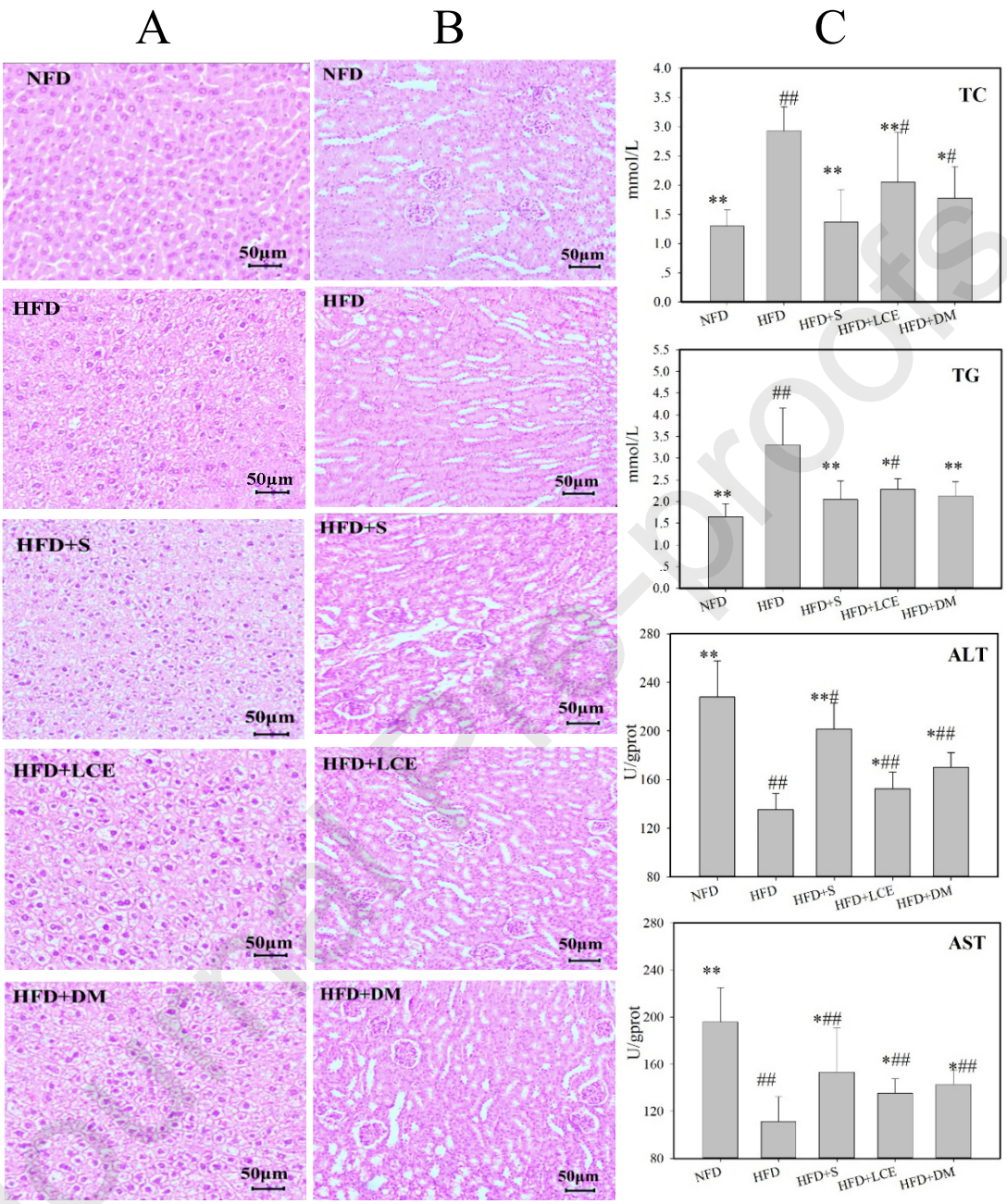
629 **Figure 5**



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632 ## Table

633 **Table 1** Impact of NFD, HFD, Simvastatin, TMP and LCE supplementation on
634 alteration of body and organ weights of KM mice (means ± SD, n=8)

Group	NFD	HFD	Δa (%)	HFD+S	Δb1 (%)	Δb2 (%)	HFD+LCE	Δc1 (%)	Δc2 (%)	HFD+DM	Δd1 (%)	Δd2 (%)

Weight (g)												
Initial	20.42 ±0.79	20.49 ±0.55	0.34	20.36 ±0.66	-0.29	-0.60	20.99 ±1.13	2.70	2.38	20.54 ±0.50	0.58	0.24
Final	40.73 ±2.67	46.66 ±2.63	12.70	38.28 ±2.81	-6.40	-21.89	37.69 ±2.17	-8.07	-23.80	37.64 ±2.07	-8.21	-23.96
Gain	20.31 ±2.48	26.17 ±2.39	28.85	17.92 ±2.28	-11.77	-31.52	16.70 ±2.66	-17.77	-36.19	17.13 ±2.13	-15.66	-34.54
Liver	2.01± 0.18	2.64± 0.26	31.34	2.22± 0.32	10.45	-15.91	2.51± 0.22	24.88	-4.92	2.24± 0.19	11.44	-15.15
Kidney	0.41± 0.05	0.65± 0.09	58.53	0.41± 0.04	0.00	-36.92	0.41± 0.05	0.00	-36.92	0.41± 0.03	0.00	-36.92

Notes: NFD: normal-fat diet; HFD: High fat diet; HFD+S: High fat diet + 6 mg/kg B.w./Day simvastatin; HFD+LCE: High fat diet +136.5 mg/kg B.w./day *Liguticum chuanxiong* alcohol extract; HFD+DM: High fat diet +163 mg/kg B.w./day drug-loading microspheres.

Δ a difference between NFD and HFD;

Δb1 difference between NFD and HFD +S;

Δb2 difference between HFD and HFD +S;

Δc1 difference between NFD and HFD + LCE;

Δc2 difference between HFD and HFD + LCE;

Δd1 difference between NFD and HFD + DM;

Δd2 difference between HFD and HFD + DM.