JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Enhanced Oral Bioavailability and Tissue Distribution of a New Potential Anticancer Agent, *Flammulina velutipes* Sterols, through Liposomal Encapsulation

Chengxue Yi,[†] Min Fu,[†] Xia Cao,[†] Shanshan Tong,[†] Qianfeng Zheng, Caleb Kesse Firempong, Xinyi Jiang, Ximing Xu,* and Jiangnan Yu*

Department of Pharmaceutics, School of Pharmacy, Center for Nano Drug/Gene Delivery and Tissue Engineering, Jiangsu University, Zhenjiang 212013, People's Republic of China

ABSTRACT: This study innovatively investigated the anticancer effect of *Flammulina velutipes* sterols (FVSs), the *in vivo* pharmacokinetics, and the tissue distribution of FVS-loaded liposomes. The FVS consisting of mainly 54.8% ergosterol and 27.9% 22,23-dihydroergosterol exhibited evident *in vitro* antiproliferative activity (liver HepG-2, IC_{50} = 9.3 μg mL^{-1}; lung A549, IC_{50} = 20.4 μg mL^{-1}). To improve the poor solubility of FVS, *F. velutipes* sterol liposome (FVSL) was originally prepared. The encapsulation efficiency of ergosterol was 71.3 ± 0.1% in FVSL, and the encapsulation efficiency of 22,23-dihydroergosterol was 69.0 ± 0.02% in FVSL. In comparison to its two free sterol counterparts, the relative bioavailability of ergosterol and 22,23-dihydroergosterol in FVSL was 162.9 and 244.2%, respectively. After oral administration in Kunming mice, the results of tissue distribution demonstrated that the liposomal FVS was distributed mostly in liver and spleen. The drug was eliminated rapidly within 4 h. These findings support the fact that FVS, a potential nutraceutical and an effective drug for the treatment of liver cancer, could be encapsulated in liposomes for improved solubility and bioavailability.

KEYWORDS: *Flammulina velutipes, sterol, anticancer, liposome, bioavailability, tissue distribution*

INTRODUCTION

Flammulina velutipes (Curt. ex Fr.) Sing., one of the most popular edible mushrooms all over the world, has been extensively studied for its nutritional components and biological activities.[1,2] By virtue of the abundant bioactive components that *F. velutipes* contains, such as proteins, glycoproteins, and mostly studied polysaccharides, *F. velutipes* exhibits antivirus, anticancer, antioxidative, and immunomodulatory properties.[3−5] However, little attention have been paid to the smaller molecules in *F. velutipes*. Although the existence of smaller molecules, such as sterols, fatty acids, flavonoids, nucleotides, and polycompounds in *F. velutipes*, have been reported,[6] the investigations on their bioactivities have been rarely demonstrated. In this study, the *F. velutipes* sterols (FVSs) have been screened out using a lipid-raft chromatographic column (data not shown), and its anticancer activity and cancer cell growth inhibition effect were also examined. To the best of our knowledge, the sterols extracted from *F. velutipes* have hardly been reported for their potential anticancer activities.

However, FVS can only dissolve in ether, chloroform, hot ethanol, or other organic solvents but hardly dissolve in water or oil at room temperature.[7] Virtually, the low water solubility makes FVS difficult to be well-dispersed in aqueous solution and creates a barrier for effective drug release. Therefore, it is urgent to seek for a proper drug delivery system, which can improve its solubility and enhance its *in vivo* bioavailability. In our previous work,[8] a microemulsion of FVS was developed, which showed remarkably enhancement solubility of FVS. However, the large amount of surfactant in the microemulsion might bring about poor biocompatibility. Because liposome is known for its low toxicity and the ability to improve bioavailability, our group decided to investigate the possibility of encapsulating the FVS in liposomes, as reported in this study.

Liposomes have been widely used as drug carriers with desirable advantages, including improving drug solubility, enhancing bioavailability, promoting drug targeting, and strengthening therapeutic effects.[9,10] Of particular interest is the ability of liposomes to improve pharmacokinetics and drug release over non-encapsulated drugs. Sidone and co-workers revealed that the pharmacokinetic variability of liposomal agents is 2.7- or 16.7-fold greater than non-liposomal agents.[11] For poorly water-soluble drugs, substantial enhancement in bioavailability or *in vivo* efficacy has been observed following liposomal encapsulation.[12] When FVS was loaded into the hydrophobic lipid bilayers, the external hydrophilic layer may help drug delivery in the aqueous environment, eventually solving the water-solubility problem of FVS and enhancing bioavailability.

In this study, FVS was isolated and determined for chemical composition by gas chromatography−mass spectrometry (GC−MS), infrared (IR), and ^{1}H and ^{13}C nuclear magnetic resonance (NMR). To confirm the anticancer activity of FVS against human cancer cell lines compared to fluorouracil (standard anticancer agent), a methyl thiazolyl tetrazolium (MTT) assay was performed to evaluate the cell viability of the

Received: December 27, 2012
Revised: May 30, 2013
Accepted: May 30, 2013
Published: May 30, 2013

human hepatoma cell line HepG-2 and human lung adenocarcinoma cell line A549. A process to prepare *F. velutipes* sterol liposome (FVSL) was also established. The prepared FVSL was characterized for its morphology, particle size, sterol content, and encapsulation efficiency. *In vivo* pharmacokinetic studies were carried out to ascertain whether the liposomal encapsulation could enhance oral bioavailability of FVS. Besides, the tissue distribution was investigated for the accumulation of the liposomal sterols in tissues and excrement compared to free sterols.

MATERIALS AND METHODS

Materials. *F. velutipes* was kindly provided by Zhengdong Ecological Agriculture Development Center (Jiangsu, China) and air-dried at 55 °C before use. Human hepatoma cell line HepG-2 and human lung adenocarcinoma cell line A549 were obtained from Cell Bank of Academy of Science (Shanghai, China). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypsin were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Company (Grand Island, NY). Fluorouracil was supplied by Jin Yao Amino Acid Co., Ltd. (Tianjin, China). Ergosterol of 98% purity was bought from Acros Organics (Geel, Belguim). Ergine of 98% purity was purchased from Xili Biotechnology Co., Ltd. (Yunnan, China). *n*-Hexane, cholesterol, sodium cholate, absolute ethanol, HCl, phosphate-buffered saline, sodium dodecyl sulfate (SDS), and isopropyl myristate were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Soybean lecithin was purchased from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Ethanol of 95% purity was purchased from Guangyuan Co., Ltd. (Shandong, China). Chromatographically pure methanol was obtained from Hanbon Technology Co., Ltd. (Jiangsu, China). Analytically pure ether was purchased from Kelong Chemical Reagent Factory (Sichuan, China). Male Sprague Dawley (SD) rats (200 ± 20 g) and Kunming mice (20 ± 2 g) were obtained from the Laboratory Animal Centre of Jiangsu University. The animals were housed under a 12 h light/dark cycle throughout the experiment at a controlled temperature of 22 ± 2 °C. They were fed with their standard food pellets and water *ad libitum* under standard laboratory conditions for 3 days. However, the animals were fasted but allowed free access to water for 12 h before the experiment. The guidelines on experiments involving the use of animals issued by the Ethic Committee of Jiangsu University were strictly followed.

Extraction of FVS. Pure FVS was obtained according to the method that we established before.[8] Briefly, dried *F. velutipes* powder (0.7 kg) was extracted 2 times with 1 L of 95% ethanol in an 85 °C water bath for 2 h. The pooled extract was concentrated using a rotary vacuum evaporator to obtain the residue. The residue was suspended in water and poured into a separating funnel. Ether was then added to it and shaken thoroughly for some time to obtain an emulsion. The mixture was allowed to settle for 20 min to obtain the ether extract. The ether extraction process was repeated 4 times. The resulting extract was then pooled together and concentrated under vacuum to give the ether residue. The ether residue was then dissolved in 1 mol L^{-1} KOH solution (using 95% ethanol as solvent for KOH) and heated with stirring to reflux for 3 h. The end product was concentrated by vacuum evaporation, and the residue was resuspended in water. The suspended residue was poured back into the separating funnel. The ether extraction process was repeated as stated above to obtain a much more refined ether residue. The ether extract was repeatedly washed with some amount of distilled water to a pH of 7.0, sufficiently shaken and washed with KOH aqueous solution (0.5 mol L^{-1}) 3 times, and finally washed with distilled water to obtain a pH of 7.0. The mixture was dehydrated with anhydrous sodium sulfate and filtered through a 0.22 μm membrane. The ether was then vacuum-evaporated to obtain a crude sterol extract. The crude sterol extract was dissolved in an appropriate amount of hot absolute ethanol, precipitated at 4 °C, and filtered to obtain pure FVS.

Component Analysis of FVS. GC−MS (Varian, Palo Alto, CA) was employed to analyze the components of FVS. *n*-Hexane was used to dissolve FVS for GC−MS determination. The GC−MS conditions were as follows: GC condition, DB-5 ms (30.0 m × 250 μm × 0.25 μm; carrier gas, high-purity helium); flow rate, 1.0 mL min^{-1}; injection port temperature, 300 °C; initial column temperature, 240 °C; column temperature, 285 °C (kept for 30 min) at the rate of 15 °C min^{-1}; and injection volume, 10 μL. MS conditions: ionization source, positive electron ionization (EI+); ionization energy, 70 eV; temperature of the ionization source, 200 °C; transfer line temperature, 290 °C; and quality scan range, 40−660 amu. To separate the sterol monomers, the purified sterol extract was dissolved in absolute methanol to obtain 1 mg mL^{-1} methanol solution and analyzed by preparative reversed-phase high-performance liquid chromatography (HPLC). The HPLC condition was as follows: Shimadzu Shim-pack PRC-ODS (20 mm × 25 cm); absolute methanol; flow rate, 5.0 mL min^{-1}; detection UV wavelength, 282 nm; and column temperature, 25 °C. The sterols were collected manually from the beginning to the end of the appearance of each peak. The fractions collected were rotary-evaporated to dryness and stored at 4 °C before use. The run time of the HPLC method was 55 min. No guard column was used. The compounds obtained from preparative HPLC were then analyzed by IR and ^{1}H and ^{13}C NMR to obtain their accurate structures. FVS (2 mg) and potassium bromide were incorporated, ground, and pressed into a pellet. Spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} on a Fourier transform infrared (FTIR) spectrometer (Thermo Nicolet Avatar 370, Thermo Fisher Scientific, Waltham, MA). The FVS was dissolved in $CDCl_3$ for NMR investigation. The ^{1}H and ^{13}C NMR spectra were recorded at 27 °C on a spectrometer (AV-500, Bruker, Germany) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. The chemical shifts were given in δ (ppm), and the delay time was 2 s.

***In Vitro* Growth Inhibition Assay.** Human liver and lung cancer cells lines HepG-2 and A549 were kept in DMEM (Sigma, St, Louis, MO) supplemented with 10% new born calf serum (Gibco, Germany) in culture flasks at 37 °C in a 5% humidified CO_2 incubator. The culturing of the cancer cells was as described by Deng et al.[13] Cells at an exponential growth phase were trypsinized and centrifuged at 400*g* for 5 min. The cells were subcultured at lower numbers (2×10^4 cells mL^{-1}) in new culture flasks. A total of 100 μL of each cell suspension (HepG-2 cells/A549 cells) was separately seeded into the 96-well culture plates (5×10^4 cells $well^{-1}$). When cells were cultured to 80% confluence, 20 μL of the FVS (0.1, 1.0, 5.0, 10.0, and 20.0 μg mL^{-1}) dissolved in 1% dimethyl sulfoxide (DMSO) was added to each row of wells. Similarly, 20 μL of 1% DMSO to make the final concentration of 0.05% as a negative control and 20 μL of fluorouracil (20.0 μg mL^{-1}) as a positive control were also used in place of the test sample for this study. When FVS/DMSO/fluorouracil treatments were added, fresh media were provided. Subsequently, 20 μL of MTT (5.0 mg mL^{-1}) was added to the cells at various time points (24, 48, and 72 h) independently, and 4 h later, the MTT-containing medium was removed and 100 μL of solubilization absolute DMSO was added. All determinations were performed in triplicate. DMSO is used to dissolve the formazan crystals that have been formed by living cells. The absorbance was measured at 570 nm using a microplate reader (Spectra III, SLT Instruments, Kernenmunster, Austria). The measured absorbance was normalized to the absorbance of non-treated control cells. The cell proliferation inhibition rate (IR, %) was calculated according to the following equation IR (%) = $(1 - A/A') \times 100\%$. A refers to the absorbance of the treated group, and A' refers to the absorbance of the untreated group. The concentration of the samples that killed 50% of the cells (IC_{50}) was calculated by CurveExpert 1.3 (Daniel G. Hyams, Hixson, TN).

During the investigation of which one of the two main components had stronger anticancer effects, an equal amount (20 μL, 20.0 μg mL^{-1}) each of ergosterol and 22,23-dihydroergosterol were used to treat HepG-2 cells. The inhibition rates at 24, 48, and 72 h were measured and calculated according to the MTT method described above. Besides, in ascertaining whether there was any synergistic effect of ergosterol and 22,23-dihydroergosterol in FVS, 20 μL each of 20.0 μg mL^{-1} FVS, 11 μg mL^{-1} ergosterol, 5.6 μg mL^{-1} 22,23-

dihydroergosterol, and a sterol mixture (consisting of 11 μg mL^{-1} ergosterol and 5.6 μg mL^{-1} 22,23-dihydroergosterol) were used to treat HepG-2 cells. Similarly, the inhibition rate was also determined, as mentioned above.

Preparation of FVSL. FVSL was prepared according to the method reported by Chu et al.,[10] with some slight modification. In brief, FVS (0.1 g) and phospholipid (1.2 g, soybean lecithin, for injection, with a phosphatidylcholine content of 70%) were placed in a single-neck flask and dissolved in 20 mL of absolute ethanol. The mixture was agitated and treated with ultrasound until the solution is clear and transparent. The solution was rotary-evaporated using a rotary evaporator (Heidolph Co., Germany) to dryness before cholesterol (0.2 g), sodium cholate (0.8 g), and isopropyl myristate (0.8 g) were added and dissolved in 20 mL of ethanol. The evaporation process was again repeated to remove ethanol, leaving film-like complexes at the bottom of the flask, which was placed in a vacuum drier for several hours to further remove residual solvent. Double distilled water was added to the dried solid to obtain a 20 mL mixture. The resulting mixture was then agitated and mixed to obtain 5 mg mL^{-1} liposomal solution. The liposomal solution was filtered through a 0.22 μm membrane to obtain FVSL solution, which was stored at 4 °C.

Morphology, Particle Size, ζ Potential, and Encapsulation Efficiency of FVSL. A total of 20 μL of 5 mg mL^{-1} FVSL solution was dyed by 2% phosphotungstic acid, dropped to screen mesh, and dried at room temperature to form a thin film. The film was then observed by transmission electron microscopy (TEM, JEM-2100, JEOL, Tokyo, Japan). A total of 3 mL of 5 mg mL^{-1} FVSL solution was measured for the particle size and distribution with a particle size analyzer (BI-9000, Brookhaven, Upton, NY). The ζ potential of 5 mg mL^{-1} FVSL was determined using a zeta plus analyzer (Zetasizer, Malvern Nano ZS, U.K.). The percentage of FVS encapsulated within the liposomes was measured according to the method described by Chu et al.,[10] with slight modification. A suitable quantity of liposomes being loaded with 100 mg of FVS was reconstituted with double distilled water to obtain a 20 mL solution. The liposomal preparation was poured on a 0.22 μm cellulose nitrate membrane to remove the unentrapped FVS. The "free" FVS was retained in the membrane, while the filtrate containing the homogeneous suspension of FVSL was collected and analyzed by HPLC. The chromatographic conditions were as follows: Shimadzu (150 × 4.6 mm, 5 μm), 98% methanol, 1.0 mL min^{-1}, 282 nm ultraviolet (UV) detector, and room temperature. The run time of the HPLC method was 55 min. No guard column was used. Reference sterols (as external standards), including ergosterol and 22,23-dihydroergosterol, were employed for the determination of the sterol content in the preparations. The free FVS, liposomal FVS, and external standards were diluted in the 98% methanol mobile phase for HPLC injection. HPLC was then used to measure the total content of ergosterol in the samples based on which the encapsulation efficiency (EE, %) was calculated according to the following equation: EE (%) = (C_1/C_2) × 100%. C_1 represented for the content of ergosterol in FVSLs, and C_2 stood for the content of ergosterol in FVSs.

***In Vitro* Release of FVSL.** The *in vitro* release tests were carried out in triplicate based on the method described in *China Pharmacopoeia* (2010 edition, paddle method), with some modifications. A total of 5 mg mL^{-1} (referring to just the FVS content) of the FVS suspension and FVSL solution was prepared as samples for determination. A total of 1.0 mL of sample solution were taken and packed into a dialysis bag (MV 3500D, 25 mm × 5 m, Shanghai Green Bird Science and Technology Development, China). The dialysis bags were fixed on the paddles before immersed into the dissolution medium (200 mL of HCl at pH 1.2 and 200 mL of phosphate-buffered saline at pH 7.4) containing 1% SDS as a surfactant. The test was performed on a dissolution tester (ZRS-8G, Tianjin University Radio Power Station, China) at 37 °C with a constant speed of 100 revolutions min^{-1}. At time points of 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, and 24 h, 2 mL of the medium was withdrawn and an equal volumn of fresh medium was added. The samples of each time points were filtered through 0.22 μm cellulose nitrate membranes and determined by the HPLC method described in the Morphology, Particle Size, ζ Potential, and Encapsulation Efficiency of FVSL section.

***In Vitro*–*in Vivo* Correlation.** According to the results determined by Akaike's information criterion method using software BAPP 2.3 (Center of Drug Metabolism of China Pharmaceutical University, China), the plasma concentration–time data for ergosterol and 22,23-dihydroergosterol released from FVSL fit into a one-compartment model. Therefore, the Wagner–Nelson method was employed in the present study to calculate the *in vivo* cumulative release. The *in vitro* release in the medium of both artificial gastric fluid and artificial intestinal fluid containing 1% SDS was used also to calculate the *in vitro*–*in vivo* correlation.

Oral Pharmacokinetics of FVSL in Rats. A total of 10 SD rats were divided equally into two groups for the oral pharmacokinetics of FVSL. A total of 5 mg mL^{-1} each of FVSL and free FVS (5 mg/mL concentration refers to just the FVS content in both solutions) was used for the oral administration with a dosage of 20 mL kg^{-1}, that is, 100 mg kg^{-1}. Free FVS was suspended in 0.5% (w/v) sodium carboxymethylcellulose (CMC–Na) solution. A total of 0.5 mL of the eye blood samples was taken at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 24 h after oral administration via gavage. The blood samples were placed in heparinized tubes and centrifuged for 10 min at 3000*g* to separate the plasma from the cellular components. A total of 5.96 mg of ergine (internal standard) was accurately weighed and dissolved in absolute methanol to obtain a solution of 19.867 μg mL^{-1}. A total of 0.1 mL of the internal standard solution was added to 0.2 mL of plasma and well-mixed prior to the addition of 1 mL of absolute methanol for protein precipitation. After vortex mixing for 5 min and centrifugation at 1500*g* for 10 min, the supernatant was nitrogen-dried in a 40 °C water bath. The residue was dissolved in 0.2 mL of the mobile phase and centrifuged at 20000*g* for 10 min. A total of 20 μL of the supernatant was injected for HPLC determination. The chromatographic conditions for biosample determination were as follows: Inertsil ODS-SP (250 × 4.6 mm, 5 μm); 96% methanol; 1.0 mL min^{-1}; 282 nm of UV detector; and 0.02 AUFS of detection sensitivity. Plasma concentrations of the various samples at different time points were calculated through the standard curves, which were obtained from the following procedure: An appropriate amount of reference sterols (ergosterol and 22,23-dihydroergosterol) was dissolved in absolute methanol to obtain a mixture of 606.4 μg mL^{-1} ergosterol and 610.4 μg mL^{-1} 22,23-dihydroergosterol as the reference stock. The stock solution was subsequently diluted into different concentrations of the sterols [ergosterol (A) and 22,23-dihydroergosterol (B)] as follows: A, 12.128, 4.852, 1.212, 0.582, and 0.348 μg mL^{-1}; B, 12.208, 4.883, 1.221, 0.586, and 0.352 μg mL^{-1}. A total of 5.96 mg of the internal standard (ergine) was dissolved in methanol to prepare a solution of 19.867 μg mL^{-1}. After that, 0.1 mL each of the diluted reference solutions with different concentrations and 0.1 mL of ergine were added to 0.2 mL of blank plasma (untreated rats) and treated as described to prepare a final solution as follows: A, 3.032, 1.213, 0.303, 0.146, and 0.087 μg mL^{-1}; B, 3.052, 1.221, 0.305, 0.146, and 0.088 μg mL^{-1}. The samples were injected into HPLC for determination. The standard curve equations, linear ranges, and regression coefficients were also calculated on the basis of HPLC results, setting the peak area ratio of the two reference sterols each to the internal standard as the vertical axis (y) and the concentration of sterol samples as the horizontal axis (x). The pharmacokinetic parameters of the drug, namely, the peak concentration in plasma (C_{max}), the time to attain the peak concentration (T_{max}), half-life time ($t_{1/2}$), and the area under the concentration–time curve ($AUC_{0-24\ h}$), were derived using BAPP 2.3 pharmacokinetic software (supplied by the center of drug metabolism of China Pharmaceutical University, China).

Tissue Distribution Study in Mice. A total of 30 Kunming mice were randomly divided into two groups. The mice were orally administrated with 20 mg kg^{-1} each of FVS and FVSL. At each time point of 1, 2, and 4 h after the administration, five mice were successively sacrificed for their tissues (including hearts, livers, spleens, lungs, kidneys, stomachs, and brains). All of the related tissues were wholly collected, except for liver (0.3 g of liver was collected). The

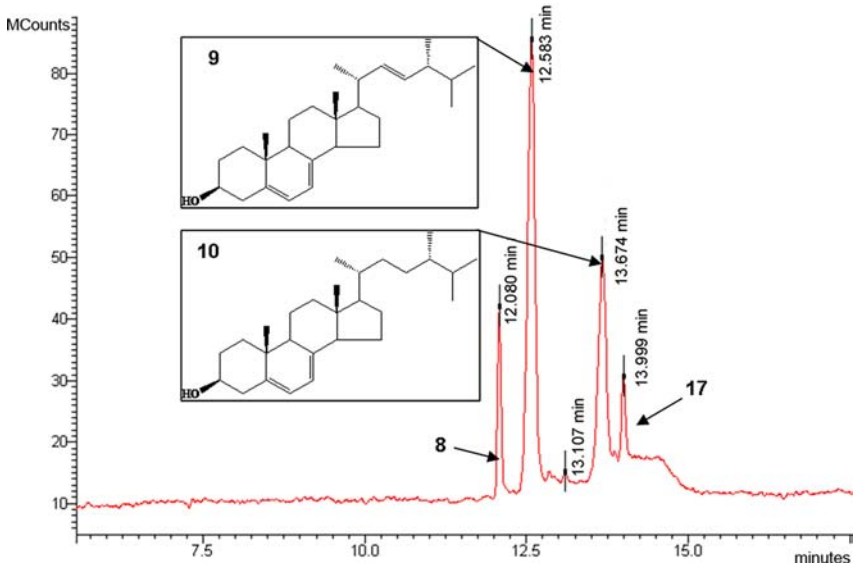

Figure 1. Total ion chromatograms of FVS, with the most predominant being ergosterol and 22,23-dihydroergosterol.

Table 1. EI−MS Data of FVS Dissolved in *n*-Hexane for GC−MS Determination

peaks	*m*/*z*	intensity (%)	assignment
ergosta-5,8,22-triene-3β-ol (**8**)	396	31	M^+
	363	100	$M^+ - H_2O - CH_3$
	337	15	$M^+ - H_2O - CH_3 - C_2H_2$
	271	31	$M^+ - SC$
	253	46	$M^+ - SC - H_2O$
ergosta-5,7,22-triene-3β-ol (**9**)	396	60	M^+
	363	100	$M^+ - H_2O - CH_3$
	339	40	$M^+ - H_2O - CH_3 - C_2H_2$
	271	20	$M^+ - SC$
	253	40	$M^+ - SC - H_2O$
ergosta-5,7-diene-3β-ol (**10**)	399	30	M^+
	365	100	$M^+ - H_2O - CH_3$
	341	50	$M^+ - H_2O - CH_3 - C_2H_2$
	271	10	$M^+ - SC$
	253	20	$M^+ - SC - H_2O$
ergosta-8(14)-ene-3β-ol (**17**)	400	67	M^+
	367	33	$M^+ - H_2O - CH_3$
	273	33	$M^+ - SC$
	255	100	$M^+ - SC - H_2O$

excrements were collected as well. Saline was added to tissues and excrements with the ratio of 0.2 g/2 mL and homogenized. The homogenizer was B25 high-shear dispersing emulsifier homogenizing machine (BRT Technology, Shanghai, China). The homogenization was performed in an ice bath 3 times with each time at 30 s. A total of 0.1 mL of internal standard solution was added to 0.5 mL of tissue and excrement homogenates. The mixture was well-mixed before 4 mL of ether was immediately added, vortex-mixed for 5 min, and centrifuged for 10 min at 1500*g*. The supernatant was dried with nitrogen in a 40 °C water bath. The residue was dissolved with 0.2 mL of the mobile phase and centrifuged for 10 min at 20000*g*. A total of 20 μL of the supernatant obtained was injected for HPLC determination. The HPLC condition was the same as described in the pharmacokinetic study, except that the mobile phase was 93% methanol for stomach samples. The sterol concentrations at different time points were calculated according to the standard curves; therefore, the weight of ergosterol and 22,23-dihydroergosterol could be obtained. The standard curves were calculated from the following procedures: The stock solution of reference sterols (ergosterol and 22,23-dihydroergosterol) was diluted in different concentrations of the sterols [ergosterol (A) and 22,23-dihydroergosterol (B)] as follows: stomach and excrement homogenate (A, 97.024, 72.768, 48.512, 24.256, 4.851, 1.213, and 0.582 μg mL^{-1}; B, 97.664, 73.248, 48.832, 24.416, 4.883, 1.221, and 0.586 μg mL^{-1}) and heart, liver, spleen, lung, kidney, and brain homogenate (A, 72.768, 48.512, 24.256, 4.851, 1.213, and 0.582 μg mL^{-1}; B, 73.248, 48.832, 24.416, 4.883, 1.221, and 0.586 μg mL^{-1}). After that, 0.1 mL each of the diluted reference solutions with different concentrations and 0.1 mL of 19.867 μg mL^{-1} ergine were added to 0.5 mL of blank tissue (untreated mice) and excrement homogenate (untreated mice) and treated as described to prepare a final solution as follows: stomach and excrement homogenate (A, 13.861, 10.395, 6.930, 3.465, 0.693, 0.173, and 0.083 μg mL^{-1}; B, 13.952, 10.464, 6.976, 3.488, 0.698, 0.174, and 0.084 μg mL^{-1}) and heart, liver, spleen, lung, kidney, and brain homogenate (A, 10.395, 6.930, 3.465, 0.693, 0.173, and 0.083 μg mL^{-1}; B, 10.464, 6.976, 3.488, 0.698, 0.174, and 0.084 μg mL^{-1}). The samples were injected into HPLC for determination. The standard curve equations, linear ranges, and regression coefficients were also calculated on the basis of HPLC results, setting the peak area ratio of the two reference sterols each to

dx.doi.org/10.1021/jf3055278 | *J. Agric. Food Chem.* 2013, 61, 5961−5971

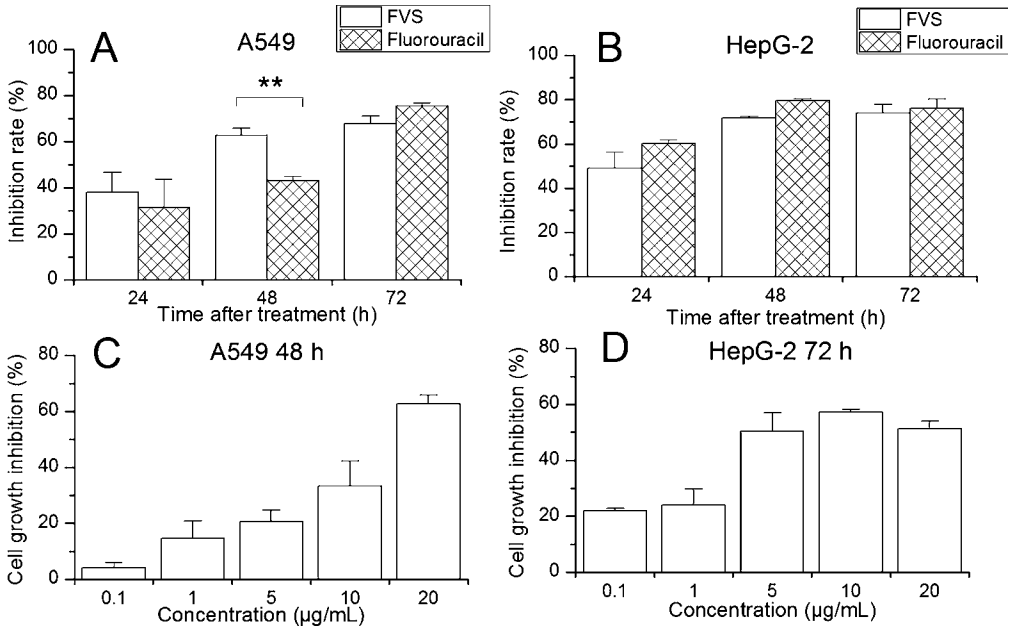

Figure 2. Human cancer cell lines susceptibility to FVS: (A and B) Antiproliferative rates of 20 μg mL^{-1} FVS and fluorouracil against A549 and HepG-2 cells at 24, 48, and 72 h and (C and D) percent cell viability measurement compared to the control at 48 h (A549) and 72 h (HepG-2) with MTT conversion for the indicated concentrations of FVS [mean + standard error of the mean (SEM); n = 3; and (**) $p < 0.01$].

the internal standard as the vertical axis (y) and the concentration of sterol samples as the horizontal axis (x).

RESULTS AND DISCUSSION

Chemical Composition Analysis of FVS. The total ion chromatogram of the FVS dissolved in *n*-hexane was analyzed in combination with the National Institute of Standards and Technology (NIST) and Wiley MS data retrieval to determine its chemical composition (Figure 1 and Table 1). The match of data revealed that, for the extraction method adopted in this study, ergosta-5,8,22-triene-3β-ol, ergosta-5,7,22-triene-3β-ol, ergosta-5,7-diene-3β-ol, and ergosta-8(14)-ene-3β-ol were obtained. The area normalization method was employed to determine the relative content of each component. The relative content of ergosta-5,8,22-triene-3β-ol, ergosta-5,7,22-triene-3β-ol, ergosta-5,7-diene-3β-ol, and ergosta-8(14)-ene-3β-ol in FVS was 11.27, 54.78, 27.94, and 5.24%, respectively. Conspicuously, the two components constituting most of FVS were ergosta-5,7, 22-triene-3β-ol (ergosterol) and ergosta-5,7-diene-3β-ol (22,23-dihydroergosterol). It is obvious that ergosta-5,7,22-triene-3β-ol and ergosta-5,7-diene-3β-ol are the major components in FVS. These two major sterols were obtained from preparative HPLC and named as compounds A and B, respectively. There was 0.05 g of compound A from 0.1 g of FVS, and 0.02 g of compound B from 0.1 g of FVS. Morphologically, compounds A and B are both needle-like crystals. The results of these compounds for the Liebermann–Burchard and Salkowshi reactions were both positive, indicating the potential existence of sterols. IR and ^{1}H and ^{13}C NMR are the commonly used methods for the analysis of chemical composition, and the results are shown as follows. Compound A: IR (KBr) υ_{max} (cm^{-1}): 3422, 2955, 2870, 1655, 1459, 1369, 1056, 835. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 5.57 (1H, dd, J = 5.5, 2.8 Hz, H-6), 5.38 (1H, ddd, J = 5.5, 2.8, 2.8 Hz, H-8), 5.26 (1H, dd, J = 15.3, 7.0 Hz, H-23), 5.19 (1H, dd, J = 15.3, 8.0 Hz, H-22), 3.6 (1H, m, H-3), 1.01 (3H, d, J = 6.5 Hz, CH_3-21), 0.94 (3H, s, CH_3-19), 0.91 (3H, d, J = 6.8 Hz, CH_3-28), 0.83 (3H, d, J = 6.0 Hz, CH_3-26), 0.80 (3H, d, J = 6.0 Hz, CH_3-27), 0.63 (3H, s, CH_3-18). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 141.3 (C-8), 139.8 (C-5), 135.6 (C-22), 132.1 (C-23), 119.6 (C-6), 116.4 (C-7), 70.5 (C-3), 55.9 (C-17), 54.6 (C-14), 46.4 (C-9), 42.9 (C-13), 42.8 (C-24), 40.9 (C-4), 40.3 (C-20), 39.2 (C-12), 38.4 (C-1), 37.1 (C-10), 33.1 (C-25), 32.0 (C-2), 28.2 (C-16), 23.0 (C-15), 21.2 (C-11), 21.1 (C-21), 19.9 (C-26), 19.6 (C-27), 17.6 (C-28), 16.3 (C-19), 12.1 (C-18). Compound B: IR (KBr) υ_{max} (cm^{-1}): 3396, 2957, 2871, 1655, 1464, 1380, 1056, 832. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 5.57 (1H, J = 3.25 Hz, H-6), 5.39 (1H, J = 2.75 Hz, H-7), 5.08 (1H, br, −OH), 3.62 (1H, H-3), 2.89 (1H, H-4), 2.46 (1H, H-4), 2.08 (1H, J = 11.0 Hz, H-12), 1.95 (1H, H-9), 1.95−1.85 (5H, H-1, H-2, H-14, H-16), 1.80−1.58 (5H, H-11, H-15, H-25), 1.48−1.16 (8H, H-1, H-15, H-16, H-17, H-20, H-22, H-23, H-24), 1.13−0.94 (8H, H-19, H-21, H-22, H-23), 0.86 (3H, d, H-27), 0.79 (3H, J = 2.6 Hz, H-26), 0.78 (3H, J = 2.6 Hz, H-28), 0.62 (3H, s, H-18). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 141.4 (C-8), 139.8 (C-5), 119.6 (C-6), 116.3 (C-7), 70.5 (C-3), 55.9 (C-17), 54.5 (C-14), 46.3 (C-9), 43.0 (C-13), 40.9 (C-4), 39.3 (C-12), 39.2 (C-24), 38.4 (C-1), 37.1 (C-10), 36.6 (C-20), 33.8 (C-22), 32.1 (C-2), 31.6 (C-25), 30.8 (C-23), 28.0 (C-16), 23.1 (C-15), 21.2 (C-11), 20.5 (C-26), 19.0 (C-21), 17.7 (C-27), 16.3 (C-19), 15.6 (C-28), 11.8 (C-18).

Thus far, researchers have found out 16 kinds of sterols extracted from *F. velutipes*:[14−17] (22*E*,24*R*)-ergosta-7,22-diene-3β,5α,6α,9α-tetrol (**1**), (22*E*,24*R*)-ergosta-7,22-diene-3β,5α,6β-triol (**2**), (24*S*)-ergosta-7-ene-3β,5α,6β-triol (**3**), 5α,8α-epidioxy-(22*E*,24*R*)-ergosta-6,22-diene-3β-ol (**4**), 5α,8α-epidioxy-(24*S*)-ergost-6-ene-3β-ol (**5**), (22*E*,24*R*)-ergosta-5,7,9(11),22-tetraene-3β-ol (**6**), (24*S*)-ergosta-7-ene-3β-ol (**7**), ergosta-5,8,22-triene-3β-ol (**8**), ergosta-5,7,22-triene-3β-ol (**9**), ergosta-5,7-diene-3β-ol (**10**), ergosta-3-*O*-β-D-glucopyranoside (**11**), ergosta-7,22-diene-5,6-epoxy-3-ol (**12**), ergosta-4,6,8(14),22-tetraene-3-one (**13**), 5α-stigmastan-3,6-dione (**14**), (24*R*)-stigmast-4-ene-3-one (**15**), and ergosta-7,22-diene-3β-ol (**16**). Moreover, we discovered ergosta-8(14)-ene-

dx.doi.org/10.1021/jf3055278 | J. Agric. Food Chem. 2013, 61, 5961−5971

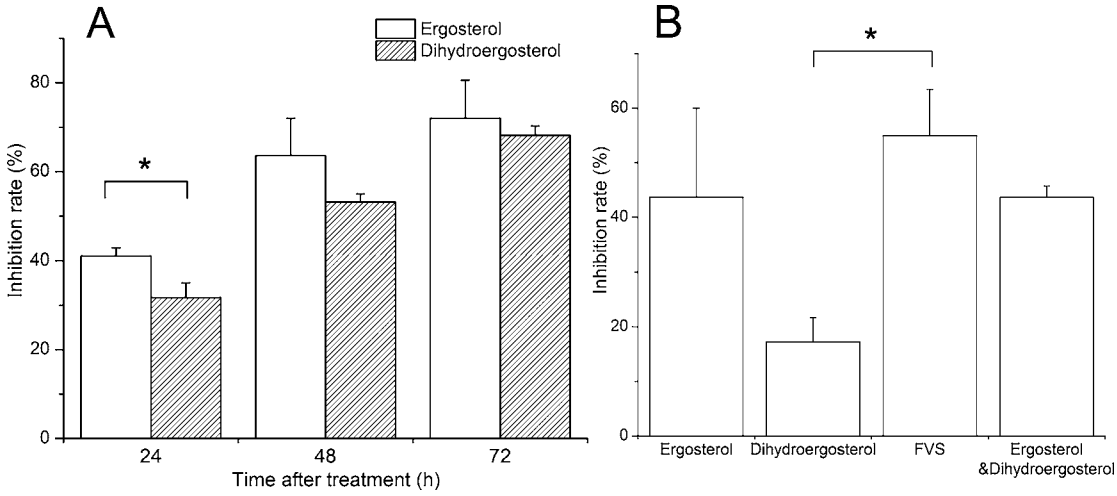

Figure 3. Inhibition rates of (A) ergosterol (20 μg mL^{-1}) and 22,23-dihydroergosterol (20 μg mL^{-1}) against HepG-2 cells at 24, 48, and 72 h and (B) ergosterol (11 μg mL^{-1}), 22,23-dihydroergosterol (5.6 μg mL^{-1}), FVS (20.0 μg mL^{-1}), and a sterol mixture (consisting of 11 μg mL^{-1} ergosterol and 5.6 μg mL^{-1} 22,23-dihydroergosterol) against HepG-2 cells at 72 h [mean + SEM; n = 3; and (*) $p < 0.05$].

3β-ol (**17**). The match of data revealed that, for the extraction method adopted in this study, compounds **8**, **9**, and **10** were obtained. The relative content of compounds **8**, **9**, **10**, and **17** in FVS was 11.27, 54.78, 27.94, and 5.24%, respectively. According to the IR and NMR information shown above, we concluded that compound A was ergosterol, while compound B was 22,23-dihydroergosterol. The two types of sterols (ergosterol and 22,23-dihydroergosterol), which accounted for most of the FVS, were obtained by preparative HPLC. The results showed an accordance with what we concluded in GC−MS. Because ergosterol and 22,23-dihydroergosterol accounted for 54.78 and 27.94% of FVS in the chemical composition analysis, the two compounds were considered as the major components of FVS and, subsequently, the most studied.

***In Vitro* Growth Inhibition Assay.** To examine the antitumor effect of FVS, the growth inhibition assay was conducted in the two human cancer cell lines (A549 and HepG-2). Complete dose-dependent graphs, especially for the A549 cancer cell lines, were generated (Figure 2), and the IC_{50} values were calculated for the FVS extract against the two human cancer cell lines mentioned above. The results also showed that, at a concentration of 20 μg mL^{-1} FVS (over a 72 h period), the inhibition rate progressively increased from 38.1 to 67.8% in A549 and from 49.1 to 74.0% in HepG-2. This inhibition rate was even comparable to the positive standard fluorouracil (from 31.5 to 75.3% in A549 and from 60.0 to 76.0% in HepG-2). This indicates that the FVS possesses desirable anticancer activity. Similarly, the IC_{50} values of the FVS in the cancer cells (HepG-2, IC_{50} = 9.3 μg mL^{-1}; A549, IC_{50} = 20.4 μg mL^{-1}) were also comparable to that of the standard drug (HepG-2, IC_{50} = 4.5 μg mL^{-1}; A549, IC_{50} = 26.7 μg mL^{-1}). Moreover, a stronger growth suppressing effect of FVS was detected against HepG-2, which presented an IC_{50} value lower than 10 μg mL^{-1}. On the basis of the American National Cancer Institute criteria[18] for an extract being promising (IC_{50} < 30.0 μg mL^{-1}) for further studies, it can be said that the FVS extract is a potential anticancer candidate, which needs to be actively investigated. It is therefore not surprising that previous studies have supported the anticancer effect of FVS against certain human cancer cell lines.[19] Our study suggests for the first time that FVS could play a vital role in developing new strategies for the prevention and treatment of human lung and liver cancers. However, further work is needed in additional cancer cell lines to confirm the potential activity of FVS against other cancers.

As presented in Figure 3A, ergosterol exhibited a stronger anticancer effect against HepG-2 cells compared to 22,23-dihydroergosterol at 20 μg mL^{-1}. This situation was independent of the treatment duration (24, 48, and 72 h). Then, in finding out whether there were any synergistic effects between the two main components, the anticancer prospects of ergosterol, 22,23-dihydroergosterol, FVS, and a mixture of ergosterol and 22,23-dihydroergosterol were investigated on HepG-2 cells at 72 h. Additionally, the concentration of the FVS group was set at 20 μg mL^{-1}, and on the basis of this value, other concentrations for the different groups were calculated. According to the proportion of ergosterol and 22,23-dihydroergosterol in FVS (54.78 and 27.94%), the calculated concentrations for these compounds were as follows: ergosterol group, 11 μg mL^{-1}; 22,23-dihydroergosterol group, 5.6 μg mL^{-1}. The sterol mixture group containing 11 μg mL^{-1} ergosterol and 5.6 μg mL^{-1} 22,23-dihydroergosterol was prepared. As seen in Figure 3B, the sterol mixture showed almost the same antiproliferative activity as in ergosterol. Furthermore, although the FVS exhibited a stronger anticancer effect than ergosterol, there was no statistically significant difference between them. Therefore, it could be said that no obvious synergistic effect was observed between ergosterol and 22,23-dihydroergosterol.

Morphology, Particle Size, ζ Potential, and Encapsulation Efficiency of FVSL. The FVSL was transparent light yellow solution with good mobility. Similar to most poorly water-soluble drugs, FVS essentially existed as a suspension in water. The solubility of free ergosterol in FVS was very low, 6.70 × 10^{-4} mg mL^{-1} in water (HPLC, 37 °C, 72 h). Meanwhile, the solubilized amount of free dihydroergosterol in FVS at the same conditions was too low to achieve the limit of detection of HPLC. However, after encapsulated in liposome, the content of ergosterol and dihydroergosterol in FVS determined by HPLC was 2.19 and 0.78 mg mL^{-1}. FVSL appeared as a homogeneous and transparent solution. Furthermore, the results of TEM revealed that the FVSL droplets were spherical in shape and have small and uniformly

dx.doi.org/10.1021/jf3055278 | J. Agric. Food Chem. 2013, 61, 5961−5971

distributed sizes (Figure 4A). The particle size of the FVS-loaded liposomes was nanoscaled, with an average diameter of

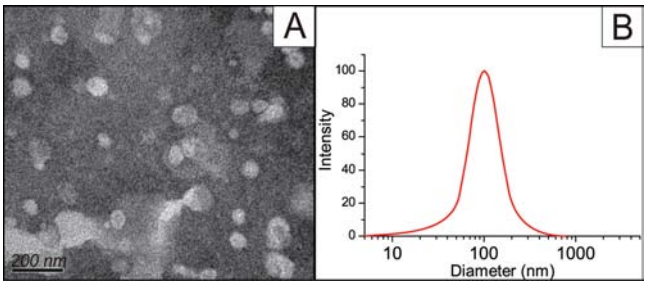

Figure 4. (A) TEM photograph and (B) particle size distribution of 5 mg mL^{-1} FVSL.

108 nm. Additionally, there was a typical normal log distribution (Figure 4B), with the polydispersity index being 0.151. As shown in Figure 4A, the particle size distribution was narrow, ranging from 68.3 to 148.6 nm. The ζ potential of 5 mg mL^{-1} FVSL was −43.3 mV, indicating that the liposome possesses desirable stability. The HPLC chromatograms of FVSL showed clear peaks representing ergosterol and 22,23-dihydroergosterol as reference sterols, while blank liposomes had no interference for the determination (data not shown). On the basis of the calculations of the external reference sterols, the loading capacities of ergosterol and 22,23-dihydroergosterol in 5 mg mL^{-1} FVSL were 2.19 and 0.78 mg mL^{-1}, respectively. The encapsulation efficiency was then calculated on the basis of the content of ergosterol in 5 mg mL^{-1} FVSL and that in free FVS suspension. According to this calculation, the encapsulation efficiency of FVSL was 71.3 ± 0.1%. If calculated with dihydroergosterol, the encapsulation efficiency would be 69.0 ± 0.02%. In comparison to the FVS microemulsion that we reported,[8] the microemulsion has a slightly higher encapsulation efficiency (81.1 ± 1.2% for ergosterol and 76.98 ± 1.45% for 22,23-dihydroergosterol). However, the liposome possesses a better loading capacity compared to the microemulsion (0.34 mg mL^{-1} for ergosterol and 0.12 mg mL^{-1} for 22,23-dihydroergosterol). The homogeneous and transparent appearance of FVSL indicated that incorporation of FVS into liposomes gave rise to enhanced solubility. This observation is in agreement with other studies, in which the liposomes have been used as drug carriers with desirable advantages, such as improving drug solubility, enhancing bioavailability, promoting drug targeting, and strengthening therapeutic effects.[9] The particle size of the liposomes (108 nm) was smaller than that of other related studies: 416 and 196.4 nm.[20,21] Our study was pleased to note that the relatively small size of these liposomes may have the potential to facilitate the oral absorption of the FVS.[10] The encapsulation efficiency of FVSL (71.3%) indicated that the bulk of FVS had been entrapped into liposomes. This efficiency value was also comparable to other related studies: 65.0 and 81.6%.[10,20] In summary, the liposomal FVS prepared in this study possesses small size, uniform size distribution, and high encapsulation efficiency. All of these properties might contribute to the improved solubility *in vitro* and enhanced absorption of FVS *in vivo*.

***In Vitro* Release of FVSL.** The *in vitro* release of FVSL was performed in both artificial gastric fluid (pH 1.2) and artificial intestinal fluid (pH 7.4) containing 1% SDS. As illustrated in Figure 5, the *in vitro* dissolutions of ergosterol and 22,23-dihydroergosterol from FVSL were both improved in

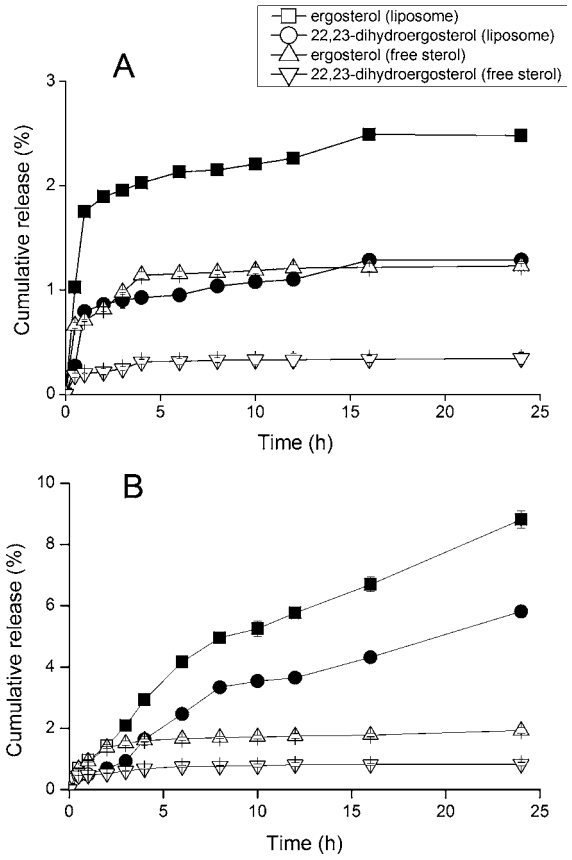

Figure 5. *In vitro* release profiles of 5 mg mL^{-1} FVS and FVSL in 200 mL of (A) HCl solution at pH 1.2 and (B) phosphate-buffered saline solution at pH 7.4, at different time points within 24 h [mean ± standard deviation (SD); $n = 3$]. Each medium contains 1% SDS. The points with significant differences ($p < 0.05$, versus free sterol) were labeled as solid.

comparison to free sterols in FVS, in either artificial gastric or intestinal fluid. The cumulative release was very low. In the artificial gastric fluid (Figure 5A), the release of ergosterol and 22,23-dihydroergosterol from FVSL accumulated to 2.48 and 1.29%, respectively, within 24 h. Generally, better release profiles were shown in the medium of artificial intestinal fluid. The cumulative release of liposomal ergosterol and 22,23-dihydroergosterol reached 8.81 and 5.81%, respectively, within 24 h (Figure 5B). However, the release rate was still very slow. Other media have been tried to seek a more appropriate medium, such as 10% ethanol solution, artificial gastric fluid containing 1% Tween 80, and artificial intestinal fluid containing 1% Tween 80. However, it turned out that the released amount of sterols was too small to be detected in these media. Only with the artificial gastric and intestinal fluid containing 1% SDS can we obtain a complete release profile. It is reasonable that the low solubility of free FVS leads to a low dissolution. As for the FVSL, the difficulty of release may be attributed to the similar structure of sterols with cholesterol. The structural similarity gave FVS a strong affinity with the cholesterol in liposome, resulting in the slow dissolution rate. However, further studies will be carried out to better investigate the behavior and mechanism of the *in vitro* release of FVSL.

***In Vitro*–*in Vivo* Correlation.** The *in vitro*–*in vivo* correlation of FVS in the artificial gastric and intestinal fluids with each containing 1% SDS is presented in Table 2. The *in vitro* release of the FVSL and free sterols in the artificial

dx.doi.org/10.1021/jf3055278 | J. Agric. Food Chem. 2013, 61, 5961−5971

Table 2. *In Vitro*–*in Vivo* Correlation of FVS in the Artificial Gastric Fluid and Artificial Intestinal Fluid with Each Containing 1% SDS

dissolution medium	preparation	sterol	regression equation	r	critical value	p
artificial gastric fluid	liposome	ergosterol	$y = 42.708x - 0.2425$	0.7797	$r(10, 0.01) = 0.708$	<0.01
		22,23-dihydroergosterol	$y = 83.009x - 0.1965$	0.6656	$r(6, 0.05) = 0.707$	>0.05
	free sterol	ergosterol	$y = 82.197x - 0.2559$	0.8387	$r(10, 0.01) = 0.708$	<0.01
		22,23-dihydroergosterol	$y = 482.98x - 0.5904$	0.7498	$r(8, 0.05) = 0.632$	<0.05
artificial intestinal fluid	liposome	ergosterol	$y = 13.367x + 0.0669$	0.9471	$r(10, 0.01) = 0.708$	<0.01
		22,23-dihydroergosterol	$y = 39.957x - 0.1014$	0.9878	$r(6, 0.01) = 0.834$	<0.01
	free sterol	ergosterol	$y = 53.382x - 0.2087$	0.8523	$r(10, 0.01) = 0.708$	<0.01
		22,23-dihydroergosterol	$y = 203.8x - 0.5993$	0.7427	$r(8, 0.05) = 0.632$	<0.05

intestinal fluid showed a desirable correlation with *in vivo* release ($p < 0.05$); however, that of the artificial gastric fluid was less effective ($p > 0.05$). Therefore, the *in vitro* release determined in the artificial intestinal fluid is likely to reflect the *in vivo* absorption.

Pharmacokinetics of FVSL in Rats. The investigation of pharmacokinetics of FVSL was carried out in rats via gavage. After oral administration of FVSL and free FVS, the plasma concentrations of ergosterol and 22,23-dihydroergosterol at different time points were detected. In the preliminary pharmacokinetic study, the sterol concentration in plasma was not able to be detected for an oral administration dose of 20 mg kg^{-1}. Therefore, the dose was increased to 100 mg kg^{-1}, which led to concentration–time profiles and pharmacokinetic parameters of both FVS-loaded liposomes and free FVS. After the oral administration, the absorption and elimination profiles of FVSL were generally much better than the free sterols. As shown in Figure 6, liposomal ergosterol exhibited a significantly

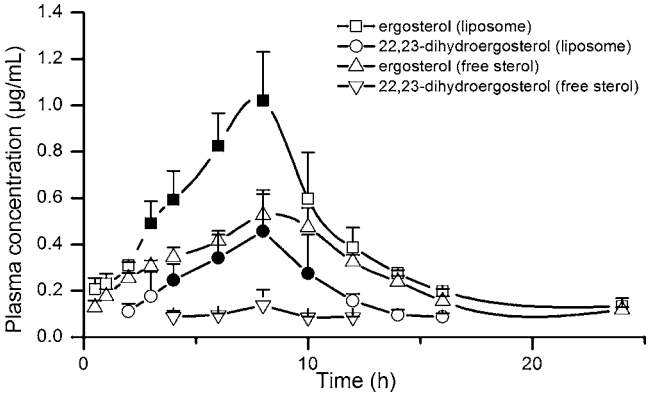

Figure 6. Mean (+SD) plasma concentration–time profile of ergosterol and 22,23-dihydroergosterol in the plasma of healthy SD rats ($n = 5$), to which a single dose of 20 mL kg^{-1} of 5 mg mL^{-1} FVSL/free FVS was orally administered. The points with significant differences ($p < 0.05$, versus free sterol) were labeled as solid.

increased plasma concentration at 3, 4, 6, and 8 h ($p < 0.05$, versus free ergosterol), while the release of liposomal 22,23-dihydroergosterol also showed a significant increase at 4, 6, 8, and 10 h ($p < 0.05$, versus free 22,23-dihydroergosterol). The plasma concentration of ergosterol and 22,23-dihydroergosterol in free sterol was at a lower and smoother level over 16 h when compared to the liposomal preparations, although a slight peak was observed at 8 h, whereas that of sterol-loaded liposomes was much greater at every time point, with a single sharp peak at a maximum concentration, which is a characteristic of non-enterohepatic circulation.[22] The pharmacokinetic parameters of sterol-loaded liposomes in plasma containing C_{max}, T_{max}, $t_{1/2}$, and $AUC_{0-24\,h}$ are presented in Table 3. The absorption of FVSL was substantially greater, as indicated by the values of C_{max} and $AUC_{0-24\,h}$. Although a bit lower than their performance in the microemulsion that we previously reported (256% for ergosterol and 450% for 22,23-dihydroergosterol),[8] it was still remarkable that the relative bioavailability of liposomal ergosterol and 22,23-dihydroergosterol was increased to 162.9 and 244.2%, respectively, in comparison to the two free sterol counterparts. A brief comparison of the liposome and microemulsion of FVS is presented in Table 4.[8] The differences between these two preparations were based on the following indices: particle size, encapsulation efficiency, loading capacity, and relative bioavailability. As seen in Table 4, the microemulsion has a smaller particle size compared to liposome, which may be helpful for the better absorption. However, the loading capacity of liposome is greater than the microemulsion. Although the encapsulation efficiency and relative bioavailability of the liposome is somehow lower than the microemulsion, the study of liposome, as a conventional dosage form, provides valuable information for FVS bioavailability, tissue distribution, and utilization.

The results of the pharmacokinetics study in rats indicated that the liposomal encapsulation could improve the absorption of FVS *in vivo* and provide a great increase in the plasma concentration, supporting other studies that also used liposomes to enhance bioavailabilities of other poorly soluble drugs.[10,21] Oral liposomes may provide increased water

Table 3. Pharmacokinetic Parameters of Free Sterol and Sterol-Loaded Liposomes in Rats (Mean ± SD; $n = 5$)

	free sterol		sterol-loaded liposomes	
parameter	ergosterol	22,23-dihydroergosterol	ergosterol	22,23-dihydroergosterol
C_{max} (μg mL^{-1})	0.53 ± 0.09	0.14 ± 0.07	1.03 ± 0.27	0.41 ± 0.17[a]
T_{max} (h)	8.00 ± 0	7.33 ± 1.15	7.33 ± 1.15	7.5 ± 1.0
$t_{1/2}$ (h)	4.75 ± 0.71	9.12 ± 4.80	4.14 ± 0.23	3.89 ± 0.60
$AUC_{0-24\,h}$ (μg h mL^{-1})	6.43 ± 0.81	1.35 ± 0.35	10.48 ± 2.58[a]	3.31 ± 1.29

[a] $p < 0.05$.

dx.doi.org/10.1021/jf3055278 | J. Agric. Food Chem. 2013, 61, 5961–5971

Table 4. Comparison of the FVS Liposome and Microemulsion Previously Reported by Our Group[8]

	liposome		microemulsion	
	ergosterol	22,23-dihydroergosterol	ergosterol	22,23-dihydroergosterol
mean particle size (nm)	108		22.9	
encapsulation efficiency (%)	71.30 ± 0.10	69.00 ± 0.02	81.1 ± 1.2	76.98 ± 1.45
loading capacity (mg mL^{-1})	2.19 ± 0.12	0.78 ± 0.02	0.34 ± 0.01	0.12 ± 0.00
relative bioavailability (%)	162.9	244.2	256.0	450.0

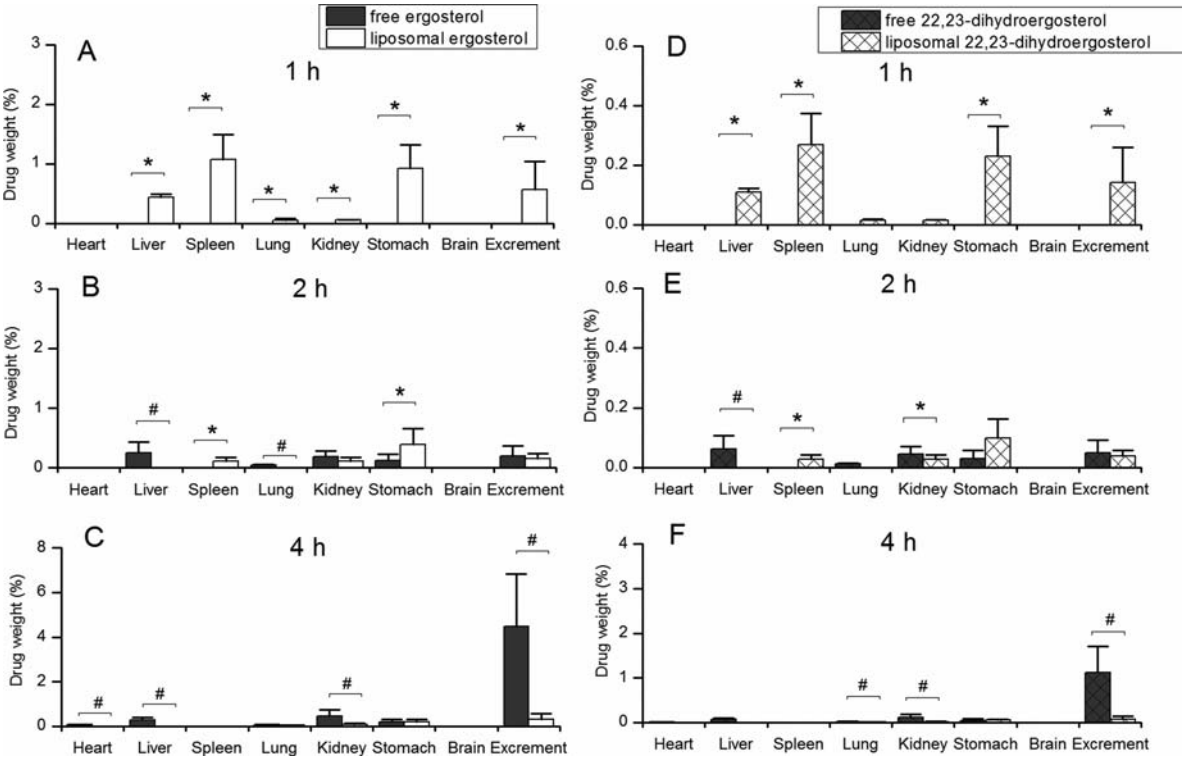

Figure 7. Tissue distribution of (A, B, and C) ergosterol and (D, E, and F) 22,23-dihydroergosterol from free FVS and FVSL at different time points (1, 2, and 4 h) in Kunming mice. Data are presented as the mean (percent drug weight of the dose) + SD; $n = 5$. (∗) $p < 0.05$; drug weight (%) in the liposomal group was significantly higher than that in the free sterol group. (#) $p < 0.05$; drug weight (%) in the liposomal group was significantly lower than that in the free sterol group.

solubility of the loaded drugs and protection from the hostile environment in the gastrointestinal tract.[23] The underlying mechanism for the facilitated oral absorption through liposomes has been partly elucidated by studies on the phase transition behavior of liposomal vesicles under a simulated gastrointestinal environment.[24] It is generally accepted that the similarity between the liposomal lipid bilayers and biomembranes and the relatively small size of liposomes significantly facilitate oral absorption.[25] However, work is currently under way to elucidate the functional mechanisms of oral bioavailability enhancement of FVSL. In conclusion, FVSL enjoys the ability to substantially increase the water solubility and bioavailability of the poorly water-soluble drug FVS, demonstrating that liposome is a desirable vessel for poorly water-soluble drugs.

Tissue Distribution Study in Mice. The investigation of tissue distribution of FVSL was conducted in mice. Using the free FVS as the negative control, the drugs were orally administrated. The tissue samples were obtained at different time points (1, 2, and 4 h) after administration, as shown in Figure 7. Generally, there would be much more ergosterol distributed in different tissues than 22,23-dihydroergosterol considering their content difference in FVS (54.78% for ergosterol and 27.94% for 22,23-dihydroergosterol). As for the tissue distribution study, in the free FVS, the sterols (<1.0 μg) were barely distributed in the different kinds of tissues for the first 2 h and basically accumulated in excrement (up to 18.0 μg) at 4 h. This was contrary to FVSL, in which the different levels of sterols were detected in most of the tissues, hence supporting other related studies.[26,27] At 1 h after oral administration, significantly higher (∗, $p < 0.05$) levels of sterol weight determined were observed in the liposomal group than the free sterol group in most tissues (panels A and D of Figure 7), indicating that FVSL was very fast and widely distributed in a short period of 1 h. The sterols in the liposomes were much more concentrated in the liver and spleen but less or none in the other related organs, such as kidney, lung, heart, and brain. After that, at the time point of 2 h, the liposomal group exhibited elimination, while the free sterol group started to distribute, thus resulting in significantly lower (#, $p < 0.05$) sterol weight for the liposomal group than the free sterol group in some specific tissues, such as liver (panels B and E of Figure 7). It was therefore not surprising that, at the end of the 4 h study period, only approximately 1.0% of the administered dose was cumulative in the excrement for the sterol-loaded liposomes. A significantly lower (#, $p < 0.05$) sterol weight

dx.doi.org/10.1021/jf3055278 | J. Agric. Food Chem. 2013, 61, 5961−5971

was observed in the liposomal group than the free sterol group, while no significantly higher sterol weight was shown any more at the time point of 4 h (panels C and F of Figure 7), which was in accordance with the fact that the liposomal sterols almost achieve complete elimination. All of the observations above indicate that the liposomal FVS could be distributed widely mostly in liver and spleen and eliminated rapidly with time, which confirms other liposomal studies.[28] In combination with the *in vitro* growth inhibition assay results, which verified the anticancer activity of FVS against liver cancer cell line HepG-2, FVSL may offer a new strategy with oral administration for the treatment of liver cancer. On the other hand, although the FVS also showed desirable anticancer activity against lung cancer cell line A549, the distribution in lung was not so satisfactory, thus requiring further experimental investigation.

In conclusion, the new potential anticancer agent FVS was successfully extracted, purified, and proven for its antiproliferative effect accordingly. A pharmacologically feasible method in the preparation of FVSL was also developed for the first time. The pharmacokinetic study in rats showed that the absorption of liposomal FVS was facilitated *in vivo* with enhanced bioavailability. In addition, the tissue distribution study suggests that the sterols in the liposomes were much more concentrated in the liver and spleen but less or none in the other related organs, such as kidney, lung, heart, and brain. These findings support that, encapsulated in liposomes for improved solubility and bioavailability, the FVS has potential to apply as a novel nutraceutical agent, most likely for the treatment of liver cancer. The observed pharmacokinetic and tissue distribution results may also be useful for evaluation of the clinical efficacy and further study of the bioactive mechanism of FVSL.

AUTHOR INFORMATION

Corresponding Author

*Telephone/Fax: +86-511-85038451. E-mail: xmxu@ujs.edu.cn (X.X.); yjn@ujs.edu.cn (J.Y.).

Author Contributions

†Chengxue Yi, Min Fu, Xia Cao, and Shanshan Tong contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant 30973677), the Doctoral Fund of Ministry of Education of China (Grant 20113227110012), and the Industry−University−Research Institution Cooperation (Grants BY2009141 and CY2010023) in Jiangsu province and Zhenjiang. The authors also thank the Jiangsu University Ethics Committee for the kind guidance in the animal experiments.

REFERENCES

(1) Fukushima, M.; Ohashi, T.; Fujiwara, Y.; Sonoyama, K.; Nakano, M. Cholesterol-lowering effects of maitake (*Grifola frondosa*) fiber, shiitake (*Lentinus edodes*) fiber, and enokitake (*Flammulina velutipes*) fiber in rats. *Exp. Biol. Med.* **2001**, *226* (8), 758−765.

(2) Ishikawa, N. K.; Yamaji, K.; Ishimoto, H.; Miura, K.; Fukushi, Y.; Takahashi, K.; Tahara, S. Production of enokipodins A, B, C, and D: A new group of antimicrobial metabolites from mycelial culture of *Flammulina velutipes*. *Mycoscience* **2005**, *46* (1), 39−45.

(3) Fu, M.; Wu, Z.; Lin, Q.; Xie, L. The purification of antiviral protein in *Flammulina velutipes* and its characteristic against tobacco mosaic virus. *J. Fujian Agric. For. Univ., Nat. Sci. Ed.* **2003**, *32* (1), 84.

(4) Jang, M. S.; Park, H. Y.; Ushio, H.; Ohshima, T. Antioxidative effects of mushroom *Flammulina velutipes* extract on polyunsaturated oils in oil-in-water emulsion. *Food Sci. Biotechnol.* **2009**, *18* (3), 604−609.

(5) Leung, M. Y.; Fung, K. P.; Choy, Y. M. The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina velutipes*. *Immunopharmacology* **1997**, *35* (3), 255−263.

(6) Lee, K. J.; Yun, I. J.; Kim, K. H.; Lim, S. H.; Ham, H. J.; Eum, W. S.; Joo, J. H. Amino acid and fatty acid compositions of *Agrocybe chaxingu*, an edible mushroom. *J. Food Compos. Anal.* **2011**, *24* (2), 175−178.

(7) Engel, R.; Schubert, H. Formulation of phytosterols in emulsions for increased dose response in functional foods. *Innovative Food Sci. Emerging Technol.* **2005**, *6* (2), 233−237.

(8) Yi, C.; Zhong, H.; Tong, S.; Cao, X.; Firempong, C. K.; Liu, H.; Fu, M.; Yang, Y.; Feng, Y.; Zhang, H. Enhanced oral bioavailability of a sterol-loaded microemulsion formulation of *Flammulina velutipes*, a potential antitumor drug. *Int. J. Nanomed.* **2012**, *7*, 5067−5078.

(9) Slingerland, M.; Guchelaar, H. J.; Gelderblom, H. Liposomal drug formulations in cancer therapy: 15 years along the road. *Drug Discovery Today* **2012**, *17* (3−4), 160−166.

(10) Chu, C.; Tong, S. S.; Xu, Y.; Wang, L.; Fu, M.; Ge, Y. R.; Yu, J. N.; Xu, X. M. Proliposomes for oral delivery of dehydrosilymarin: Preparation and evaluation in vitro and in vivo. *Acta Pharmacol. Sin.* **2011**, *32* (7), 973−980.

(11) Sidone, B.; Zamboni, B. A.; Zamboni, W. Meta-analysis of the pharmacokinetic variability of liposomal anticancer agents compared with nonliposomal anticancer agents. *ASCO Meet. Abstr.* **2011**, *29* (Supplement 15), 2583.

(12) Mourão, S. C.; Costa, P. I.; Salgado, H.; Gremião, M. P. D. Improvement of antischistosomal activity of praziquantel by incorporation into phosphatidylcholine-containing liposomes. *Int. J. Pharm.* **2005**, *295* (1), 157−162.

(13) Deng, W. W.; Cao, X.; Wang, M.; Yang, Y.; Su, W. Y.; Wei, Y. W.; Ouyang, Z.; Yu, J. N.; Xu, X. M. Efficient gene delivery to mesenchymal stem cells by an ethylenediamine-modified polysaccharide from mulberry leaves. *Small* **2012**, *8* (3), 441−451.

(14) Kang, J.; Wang, H. Q.; Chen, R. Y. Studies on the constituents of the mycelia produced from fermented culture of *Flammulina velutipes* (W. Curt.: Fr.) Singer (Agaricomycetideae). *Int. J. Med. Mushrooms* **2003**, *5* (4), 391−396.

(15) Yokokawa, H.; Mitsuhashi, T. The sterol composition of mushrooms. *Phytochemistry* **1981**, *20* (6), 1349−1351.

(16) Kang, J.; Chen, R. Y. Studies on chemical constituents in the mycelia from fermented culture of *Flammulina velutipes*. *China J. Chin. Mater. Med.* **2005**, *30* (3), 193−194.

(17) Yaoita, Y.; Amemiya, K.; Ohnuma, H.; Furumura, K.; Masaki, A.; Matsuki, T.; Kikuchi, M. Sterol constituents from five edible mushrooms. *Chem. Pharm. Bull.* **1998**, *46* (6), 944−950.

(18) Suffness, M.; Pezzuto, J. M. Assays related to cancer drug discovery. In *Methods in Plant Biochemistry: Assays for Bioactivity*; Hostettmann, K., Ed.; Academic Press: London, U.K., 1990; Vol. *6*, pp 71−133.

(19) Mizuno, T.; Saito, H.; Nishitoba, T.; KaWagishi, H. Antitumor-active substances from mushrooms. *Food Rev. Int.* **1995**, *11* (1), 23−61.

(20) Hazra, B.; Kumar, B.; Biswas, S.; Pandey, B.; Mishra, K. Enhancement of the tumour inhibitory activity, in vivo, of diospyrin, a plant-derived quinonoid, through liposomal encapsulation. *Toxicol. Lett.* **2005**, *157* (2), 109−117.

(21) Xiao, Y. Y.; Song, Y. M.; Chen, Z. P.; Ping, Q. N. The preparation of silybin−phospholipid complex and the study on its pharmacokinetics in rats. *Int. J. Pharm.* **2006**, *307* (1), 77−82.

(22) Morazzoni, P.; Montalbetti, A.; Malandrino, S.; Pifferi, G. Comparative pharmacokinetics of silipide and silymarin in rats. *Eur. J. Drug Metab. Pharmacokinet.* **1993**, *18* (3), 289−297.
(23) Ariën, A.; Goigoux, C.; Baquey, C.; Dupuy, B. Study of in vitro and in vivo stability of liposomes loaded with calcitonin or indium in the gastrointestinal tract. *Life Sci.* **1993**, *53* (16), 1279−1290.
(24) Chen, Y. P.; Lu, Y.; Chen, J. M.; Lai, J.; Sun, J.; Hu, F. Q.; Wu, W. Enhanced bioavailability of the poorly water-soluble drug fenofibrate by using liposomes containing a bile salt. *Int. J. Pharm.* **2009**, *376* (1−2), 153−160.
(25) Aungst, B. J. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. *J. Pharm. Sci.* **1993**, *82* (10), 979−987.
(26) Papahadjopoulos, D.; Gabizon, A. Targeting of liposomes to tumor cells in vivo. *Ann. N. Y. Acad. Sci.* **1987**, *507* (1), 64−74.
(27) Mishra, K. P.; Gopal, A. R.; Marathe, D.; Pandey, B. N. Prospects of temperature sensitive liposomal delivery of drug to tumour. In *Advances in Liposomal Therapeutics*; Vyas, S. P., Dixit, V. K., Eds.; CBS Publishers: New Delhi, India, 2001; pp 134−138.
(28) Zhao, Y. Y.; Cheng, X. L.; Liu, R.; Ho, C. C.; Wei, F.; Yan, S. H.; Lin, R. C.; Zhang, Y.; Sun, W. J. Pharmacokinetics of ergosterol in rats using rapid resolution liquid chromatography−atmospheric pressure chemical ionization multi-stage tandem mass spectrometry and rapid resolution liquid chromatography/tandem mass spectrometry. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2011**, *879* (21), 1945−1953.