Cytotoxicity and enhancement activity of essential oil from *Zanthoxylum bungeanum* Maxim. as a natural transdermal penetration enhancer[*]

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Abstract: The aim of this present study is to investigate the effect of *Zanthoxylum bungeanum* oil (essential oil from *Z. bungeanum* Maxim.) on cytotoxicity and the transdermal permeation of 5-fluorouracil and indomethacin. The cytotoxicity of *Z. bungeanum* oil on dermal fibroblasts and epidermal keratinocytes was studied using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The rat skin was employed to determine the percutaneous penetration enhancement effect of *Z. bungeanum* oil on hydrophilic and lipophilic model drugs, i.e., 5-fluorouracil and indomethacin. The secondary structure changes of the rat stratum corneum (SC) were determined using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), and saturated solubilities and SC/vehicle partition coefficients of two model drugs with and without *Z. bungeanum* oil were also measured to understand its related mechanisms of action. It was found that the half maximal inhibitory concentration (IC_{50}) values of *Z. bungeanum* oil were significantly lower in HaCaT and CCC-ESF-1 cell lines compared to the well-established and standard penetration enhancer Azone. The *Z. bungeanum* oil at various concentrations effectively facilitated the percutaneous penetration of two model drugs across the rat skin. In addition, the mechanisms of permeation enhancement by *Z. bungeanum* oil could be explained with saturated solubility, SC/vehicle partition coefficient, and secondary structure changes of SC.

Key words: *Zanthoxylum bungeanum* Maxim., Essential oil, Transdermal delivery, Penetration enhancer, HaCaT, Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

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1 Introduction

The pericarp of *Zanthoxylum bungeanum* Maxim., belonging to the Rutaceae family, is commonly used as a flavoring and traditional Chinese medicine for its flavors and medicative characteristics in China. According to the theory of traditional Chinese medicine (TCM), the pericarp of *Z. bungeanum* Maxim. possesses the medical functions of warming the spleen and stomach to relieve pain and killing parasites to relieve itching, and it is also effective for the therapy of pathogenic wind, epigastric pain, eczema, pruritus, fungal infection, diarrhea, and dysentery (Gong *et al.*, 2009; Wei *et al.*, 2011; Zhu *et al.*, 2011). In addition, *Z. bungeanum* oil is also widely used for topical remedies. According to the statement on *Li Yue Pian*

Wen (published in 1870), an ancient classic literature on topical remedies in China, these herbs like *Z. bungeanum* Maxim., peppermint oil, which are rich in essential oil, can effectively promote the percutaneous absorption of the active components in a prescription for their unique properties.

In our previous studies, Zhitong cataplasm, a clinical empirical formula mainly consisting of the extracts of four herbs, Pericarpium Zanthoxyli (*Z. bungeanum* Maxim.), Rhizoma Chuanxiong, Radix Angelicae Dahuricae, and Herba Asari, was developed and topically used for the treatment of rheumatoid arthritis and joint pain. We found that *Z. bungeanum* oil in this prescription had the function of facilitating the percutaneous absorption of the active components besides its medicative function as described above. However, there is currently no systematic information available about the skin permeation enhancement effect of *Z. bungeanum* oil. It is well known that it is a prerequisite to enable the drug molecule to pass through the skin for the exertion of drug pharmacological activity. Furthermore, most of the essential oil is mainly composed of terpenes (Gong *et al.*, 2009; Xia *et al.*, 2011), which have been generally reported to enable the drug molecule to penetrate across the skin as a penetration enhancer (Aqil *et al.*, 2007; Sapra *et al.*, 2008). The *Z. bungeanum* oil is thus supposed to possess the properties of facilitating the percutaneous absorption of the components in a prescription besides its medicative characteristics. In addition, the *Z. bungeanum* oil appears to offer low toxicity potential for use in transdermal formulations due to its natural origin (Fox *et al.*, 2011).

Hence, the aim of this study is to investigate the toxicity and percutaneous penetration enhancement activity of *Z. bungeanum* oil and its underlying mechanisms. Considering the diverse properties of the components penetrating through the skin, 5-fluorouracil (5-FU, *n*-octanol/water partition coefficient $\log K_{o/w}=$ −0.95) and indomethacin (IM, $\log K_{o/w}=3.80$) were chosen as hydrophilic and lipophilic model drugs, respectively (Zhao *et al.*, 2008). In addition, rat skin was employed to monitor the skin permeation enhancement effect of *Z. bungeanum* oil on the transdermal delivery of model drugs.

2 Materials and methods

2.1 Drugs and chemicals

5-FU and IM were obtained from Beijing Ouhe Technology Co., Ltd. and Suzhou Yacoo Co., Ltd., China, respectively. 1,8-Cineole, limonene, and terpinen-4-ol were supplied by the TCI Tokyo Chemical Industry Co., Ltd., Japan. Propylene glycol (PG) was purchased from Beijing Chemical Reagent Co., Ltd., China. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and C_8–C_{20} *n*-alkanes were obtained from Sigma-Aldrich (Shanghai, China). Azone was purchased from Beijing Changhua Fine Chemical Co., Ltd., China. Acetonitrile and methanol of high-performance liquid chromatography (HPLC) grade were supplied by Merck KGaA (Germany). All other chemicals used were of analytical grade.

2.2 Plant materials and essential oil extraction

The dried fruits of *Z. bungeanum* used in this work were purchased from Anguo Lulutong Co., Ltd. (Hebei, China), and identified by Prof. Shou-ying DU (Department of Chinese Pharmacy, Beijing University of Chinese Medicine, China). These specimens were harvested in August, 2012. A voucher specimen was deposited in the Department of Chinese Pharmacy, Beijing University of Chinese Medicine, China. The dried herbs (2.0 kg) were subjected to hydrodistillation in 5 volumes of water for 3 h by using a Clevenger-type apparatus. The yellowish essential oil was obtained in a yield of 2.9% (w/w) after drying with anhydrous sodium sulphate and stored under N_2 at 4 °C in a sealed brown vial until tested and analyzed.

2.3 Cell line and culture

HaCaT (epidermal keratinocytes) and CCC-ESF-1 (dermal fibroblasts) cell lines were obtained from the Cell Resource Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Peking Union Medical College, China). The cells were incubated in Minimum Essential Medium (MEM Eagles with Earle's Balanced Salts) and Dulbecco's Modified Eagle's Medium (DME H-21 4.5 g/L glucose), respectively, supplemented with

10% fetal bovine serum (FBS), 100 U/ml penicillin/ streptomycin at 37 °C in a 5% CO_2 incubator. HaCaT cells were fusiform under a microscope (Fig. 1c), and CCC-ESF-1 cells were fibroblast-like cells (Fig. 1d).

2.4 HPLC methodology of model drugs

The HPLC system for analyzing drug concentrations was equipped with an SPD-20A variable-wavelength ultraviolet absorbance detector, two LC-20AT pumps, and computer integrating system (Shimadzu, Kyoto, Japan). Alltima AQ ODS column (250 mm×4.6 mm i.d., 5 μm particle size; Grace) and Purospher STAR RP-18 column (250 mm×4.6 mm i.d., 5 μm particle size; Merck) were used to determine the contents of 5-FU and IM, respectively. For 5-FU, the mobile phase was distilled water at a flow rate of 1 ml/min. 5-FU was detected at 266 nm with the retention time of 7.5 min. For IM, the mobile phase was a mixture of acetonitrile/0.1 mol/L acetic acid (45:55, v/v) at the flow of 1 ml/min. IM was detected at 231 nm with the retention time of 12.5 min. Calibration curves for 5-FU and IM were linear over the ranges of 0.5–500 μg/ml and 0.5–250 μg/ml, respectively. The coefficients of variation for intra- and inter-day variations were below 1%.

2.5 Gas chromatography-mass spectral (GC-MS) analysis

The *Z. bungeanum* oil was subjected to GC-MS analysis on a Shimadzu system (Shimadzu QP-2010, Japan) equipped with a National Institute of Standards and Technology (NIST) database and a DB-1MS capillary column (30 m×0.25 mm×0.25 mm, Agilent). Helium (99.99%) was the carrier gas at a flow rate of 1.0 ml/min. The oven temperature was held at 50 °C for 20 min and then programmed from 50 to 200 °C at a rate of 2 °C/min. The injector temperature, MS transfer line and ion source temperatures were set at 250, 280, and 250 °C, respectively. Mass scanning was in the range of 45–500 amu. The split ratio was 1:20. An aliquot (1.0 μl) of the diluted sample (1/100 in *n*-hexane, w/v) was injected automatically.

The constituents of the essential oil were identified by matching their MS fragmentation patterns with those in NIST 2008 mass spectral library and also by comparing their Kovat retention indices, which were determined by injection of the sample with a

solution containing the homologous series of C_8–C_{20} *n*-alkanes as reported before (Sereshti *et al.*, 2011; Wu *et al.*, 2012). Moreover, the major oil contributors were further confirmed by comparing the corresponding pure standard compounds. The percentage composition was computed by the normalization method from the GC peak areas.

2.6 Determination of physicochemical properties of essential oil

For recognition of the basic physiochemical properties of *Z. bungeanum* oil, an ultraviolet-visible (UV-VIS) spectroscopy (UV-VIS) spectrophotometer (SP-752, Shanghai Spectrum Instruments Co., Ltd., China) was employed to monitor its maximum UV absorption wavelength. An automatic surface tensiometer (JK99B, Shanghai Zhongcheng Digital Technology Instruments Co., Ltd., China) was adopted to detect the surface tension of the essential oil. The conductivity of the oil was determined using a DDS-307 conductivity meter (Shanghai Jingke Electronic Co., Ltd., China). The pH value measurement was made using a pH meter (PHS-3C, Sanxin, China) at 25 °C. The relative density was measured through a weighing method using an electronic balance (Sartorius, Germany).

2.7 Preparation of full thickness skin and stratum corneum (SC)

Male Sprague-Dawley (SD) rats weighing (200± 10) g were purchased from Shibeifu Laboratory Animal Technology Co., Ltd. (Beijing, China). After sacrificing the rats with excess ether inhalation, hair from the abdominal surface was removed with an animal hair clipper (Codos, China) with an extreme precaution as to not impair the skin. The shaved skin was then excised from the animals, the subcutaneous tissue was removed surgically and the dermal side was wiped with a cotton swab to remove the adhered fat tissue. The full thickness skin prepared was subsequently washed with phosphate buffer saline (PBS), wrapped in aluminum foil, and stored at −20 °C (used within two weeks). All the animal experiments were conducted in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Beijing University of Chinese Medicine, China, and the experimental protocol was approved by the Committee on Animal Research of the Beijing University of

Chinese Medicine.

The excised rat skin was treated with 2 mol/L sodium bromide solution for 8 h as reported previously (Jain et al., 2002). The SC samples were separated by using a cotton swab moistened with double distilled water. SC sheets were thoroughly washed with water and dried in a vacuum desiccator.

2.8 Cytotoxicity assay

To determine the cellular toxicity of Z. bungeanum oil in epidermal keratinocyte and dermal fibroblast cultures, the MTT assay was used to monitor the toxic effects of Z. bungeanum oil on skin cells in vitro as described in detail elsewhere (Song et al., 2005). Fibroblasts or keratinocytes were seeded into 96-well plates at a density of 7000 cells in 100 µl medium per well. After 24 h, the cells were incubated with varying concentrations of enhancer solution in a culture medium with 1% DMSO (as a vehicle) for 24 h at 37 °C. The cells that were treated with culture medium containing 1% DMSO were used as the control. Subsequently, the medium was removed and supplemented with fresh medium containing 20 µl MTT solution (5 mg/ml in phosphate buffer) and the cells were incubated for 4 h. Then the medium was replaced with 150 µl DMSO to dissolve the formazan crystals. The plate was incubated for 10 min while shaking. Absorbance was read at 490 nm using a microplate spectrophotometer (Thermo Scientific, Finland). The half maximal inhibitory concentrations (IC_{50}) were calculated using SPSS software v16.0 according to the previously described method (Xiang et al., 2010).

2.9 Skin permeation studies

The vertical Franz-type diffusion cell (Shanghai Kaikai Technology Trade Co., Ltd., China) with a diffusional area of 1.77 cm^2 and a receptor chamber volume of 7 ml was employed to investigate percutaneous penetration enhancement activity of the Z. bungeanum oil. The epidermis prepared was sandwiched between the diffusion cells with the SC side up and the dermal side exposed to the receiver compartment containing isotonic PBS (pH 7.2), the receptor cells were thermostated at 32 °C, and the solution in the receptor chambers was stirred continuously at 300 r/min. The skin was then treated with 2 ml of varying concentrations of Z. bungeanum oil in PG:water (80:20, v/v). Saturated suspensions of model drugs were used to insure maximum thermodynamic activity and maintain sink conditions. The control was treated with a vehicle only. Samples (1 ml) were withdrawn from the receptor chamber at predetermined time (1, 2, 4, 6, 8, 10, 12, 14, 22, and 24 h), and replaced with an equivalent volume of buffer solution, and the samples were immediately assayed using the HPLC method described in Section 2.4.

2.10 Determination of saturated solubility of model drugs

In order to investigate the effect of Z. bungeanum oil applied on the saturation solubility of the model drugs, an excess drug was added to the known volumes of the vehicle with or without varying concentrations of Z. bungeanum oil, vortexed for 3 min followed by sonication for 15 min to dissolve the drug, and then equilibrated at (32±0.5) °C for more than 48 h. The saturated solution was then centrifuged at 10000 r/min for 15 min and aliquots of supernatant were filtered through a 0.45-µm nylon filter and diluted by a mobile phase before HPLC analysis.

2.11 Measurement of SC/vehicle partition coefficient of model drugs

The partition coefficient of model drugs into SC with or without various concentrations of Z. bungeanum oil treatment was measured as reported previously (Song et al., 2005). The SC samples were pulverized in a mortar with a pestle. One milliliter of varying concentrations of Z. bungeanum oil in PG:water (80:20, v/v) containing 0.1 g/ml model drug was added to 10 mg ground SC with frequent vortexing. The control was treated with a vehicle only. The mixture was equilibrated for 10 h at 37 °C. The supernatant solution was obtained by centrifuging at 10000 r/min for 10 min and then analyzed for the drug content. The amount of drug bound to the SC was calculated by subtracting the amount of the drug in the supernatant from the initial drug concentration. All partition studies were conducted in triplicate. The partition coefficient (K) of the model drug was obtained using the following equation: $K=$ (drug concentration in SC)/(drug concentration in vehicle).

2.12 Fourier transform infrared spectroscopy (FTIR) studies

The dried SC sheet was cut into approximately 1 cm^2 pieces and incubated for 12 h in 5 ml of the respective solutions at room temperature, where the solutions were 1%, 3%, 5%, and 10% Z. bungeanum oil, 0.2 mol/L Azone, and solvent (PG:water=80:20 (v/v), used as the control), respectively. The SC was then cleaned carefully with distilled water to remove the residual solvent on the SC surface and placed in a vacuum desiccator at 37 °C overnight for complete dehydration. The spectral measurements of all pieces were made with a Nexus FTIR spectrometer (Thermo Nicolet, USA) equipped with an attenuated total reflectance (ATR) attachment with the following parameters: resolution of 2 cm^{-1}, scanning times of 100, and scanning range of 650–4000 cm^{-1}. The FTIR spectral curves in each group were recorded using an OMNIC 6.2 program attached to the IR instrument itself.

2.13 Data and statistical analysis

The parameters for the skin permeation studies were calculated by plotting the cumulative amount of drug permeated across the skin against time (h). Steady state flux (J_s) was calculated as the slope of the linear portion of the plot (between 8 and 14 h). The lag time (T_{lag}) was determined by extrapolating the linear portion of the curve to the X-axis. The cumulative drug amount in the receptor chamber after 24 h (Q_{24}) and diffusion parameter (D/h^2) were calculated from the following equations:

$$Q_{24} = V_r C_t + \sum_{i=0}^{t-1} V_s C_i, \qquad (1)$$

$$D / h^2 = 1/(6T_{lag}), \qquad (2)$$

where C_i is the drug concentration of the receiver solution at each sampling time, C_t is the drug concentration of the sample, V_s and V_r are the volumes of the sampling solution and the receiver solution, respectively, D is the diffusion coefficient within the skin, and h is the diffusional path length.

In order to compare the permeation enhancement capacity of different concentrations of Z. bungeanum oil, the enhancement ratio (ER) for flux was calculated using the following equation: ER=(flux for skin treated with essential oil)/(flux for control (exposed to only vehicle)).

Data were expressed as the mean±standard deviation (SD) and the number of replicates (n) was given in the pertinent figures. A two-tailed Student's t-test was used when comparing two different conditions. In all cases, $P<0.05$ was considered significant.

3 Results and discussion

3.1 Chemical compositions and physicochemical properties of the Z. bungeanum oil

A total of 48 compounds were identified from the essential oil, which represented 96.35% of the total oil. There were 31 constituents accounting for above 0.5% of the total oil as listed in Table 1, and three major oil contributors (i.e., terpinen-4-ol, 1,8-cineole, and limonene, which represented 41.38% of the total oil) were further confirmed using the corresponding pure compounds. Based on the results of the GC-MS analysis, the Z. bungeanum oil contained high contents of oxygenated monoterpenes and monoterpene hydrocarbons, and the major compounds were terpinen-4-ol (18.42%), 1,8-cineole (15.49%), limonene (7.47%), α-terpineol (5.79%), and γ-terpinene (5.62%), which was slightly different from the previous report (Wang et al., 2006) on account of different producing areas, growth conditions, and extracted methods. The results of the measurement of the essential oil physicochemical properties exhibited that the oil had the physiochemical parameters as follows: density 0.86 mg/ml; pH 5.18; electric conductivity 0 μs/cm; surface tension 24.96 mN/m; maximum UV absorption wavelength 227 nm.

Generally, the major components determine the biological properties of the essential oil, and it has been reported that some terpenes identified from the essential oil could enhance the transdermal permeation of the polar and non-polar drugs, such as terpinen-4-ol (Magnusson et al., 1997), 1,8-cineole, terpineol (Narishetty and Panchagnula, 2005), and limonene (Okabe et al., 1989), indicating these major oil contributors play a crucial role in the enhancement activity of Z. bungeanum oil as a transdermal penetration enhancer.

Table 1 Major components of the essential oil from *Zanthoxylum bungeanum* detected by GC-MS

t_R^a (min)	Compound	RI^b	ID method	Relative content (%)
9.48	3-Thujene	917	MS, RI	0.59
9.81	α-Pinene	923	MS, RI	1.21
11.99	β-Phellandrene	957	MS, RI	1.80
13.46	Myrcene	979	MS, RI	3.89
15.17	α-Terpine	1004	MS, RI	3.29
15.39	*o*-Cymene	1007	MS, RI	1.53
16.02	1,8-Cineole	1013	MS, RI^c	15.49
16.28	Limonene	1016	MS, RI^c	7.47
17.05	(E)-β-ocimene	1024	MS, RI	1.19
17.94	(Z)-β-ocimene	1034	MS, RI	0.53
18.72	γ-Terpinene	1042	MS, RI	5.62
21.36	Terpinolene	1071	MS, RI	1.69
22.42	Linalool	1082	MS, RI	4.55
23.93	**1**	1098	MS, RI	1.10
25.67	**2**	1114	MS, RI	1.03
28.69	Cryptone	1141	MS, RI	1.72
30.08	Terpinen-4-ol	1153	MS, RI^c	18.42
31.25	α-Terpineol	1163	MS, RI	5.79
31.93	**3**	1169	MS, RI	0.66
33.20	**4**	1180	MS, RI	0.58
34.16	**5**	1189	MS, RI	0.52
35.14	Cumic aldehyde	1198	MS, RI	0.50
36.91	Piperitone	1212	MS, RI	2.62
39.25	Phellandral	1232	MS, RI	1.08
40.02	Linalyl acetate	1238	MS, RI	0.55
41.91	Cuminic alcohol	1254	MS, RI	0.52
50.42	Terpinyl acetate	1324	MS, RI	4.62
51.60	Citronellyl acetate	1334	MS, RI	0.62
54.75	Geranyl acetate	1360	MS, RI	0.68
75.73	Caryophyllene oxide	1538	MS, RI	0.57
107.84	Octadecane	1843	MS, RI	1.32
	Total			91.75

Compound **1**: *trans*-4-(isopropyl)-1-methylcyclohex-2-en-1-ol; Compound **2**: *cis*-4-(isopropyl)-1-methylcyclohex-2-en-1-ol; Compound **3**: *trans*-6-(isopropyl)-3-methylcyclohex-2-en-1-ol; Compound **4**: *cis*-6-(isopropyl)-3-methylcyclohex-2-en-1-ol; Compound **5**: (Z)-2-methyl-5-(1-methylethenyl)-cyclohexen-2-ol. [a] t_R is the retention time of the identified compounds; [b] RI is Kovat retention indices on DB-1MS column; [c] It is further confirmed by using the corresponding pure compound. ID: identification

3.2 Cytotoxicity of *Z. bungeanum* oil on skin cells

Most penetration enhancers have been shown to produce skin irritation or toxicity in spite of their fairly satisfactory performance in enhancing the permeation of drug molecules; hence few of them have been approved for clinical use. The cytotoxicities of *Z. bungeanum* oil and the well-established and standard penetration enhancer Azone were studied using MTT assay, and the results of keratinocytes and fibroblasts treated with different concentrations of selected penetration enhancers are presented in Fig. 1. All examined permeation enhancers induced dose-dependent reductions in cellular viability after 24 h (Figs. 1a and 1b). As summarized in Table 2, the IC_{50} values (i.e., the concentration of drugs inducing a 50% decrease in cell viability) of *Z. bungeanum* oil were prominently higher in both HaCaT cells and CCC-ESF-1 cells compared to those of Azone, indicating that *Z. bungeanum* oil was probably a low toxic penetration enhancer.

Table 2 IC_{50} values of Azone and the *Zanthoxylum bungeanum* oil for HaCaT and CCC-ESF-1 cell lines

Penetration enhancer	IC_{50} (mg/ml)	
	HaCaT	CCC-ESF-1
Azone	0.047±0.002	0.048±0.004
Z. bungeanum oil	2.435±0.019	3.649±0.055

Each value represents the mean±SD (*n*=6)

3.3 Effects of *Z. bungeanum* oil on percutaneous permeation of model drugs

To determine the permeation enhancement activity of *Z. bungeanum* oil across the rat skin, the control was prepared only by dissolving the model drugs in the base solvent, i.e., PG:water (80:20, v/v). PG:water was chosen as a base solvent on account of its ability to solubilize most tested components, including *Z. bungeanum* oil and Azone. Moreover, as the well-established and standard penetration enhancer, Azone had been employed by many researchers to achieve significant permeation enhancement and the concentration (0.2 mol/L) was typically used in skin penetration enhancement studies (Meidan *et al.*, 2003; Kaushik *et al.*, 2010; Batheja *et al.*, 2011); hence 0.2 mol/L Azone was used to compare and better evaluate the permeation enhancement activity of the oil.

The effect of *Z. bungeanum* oil at various concentrations (1%, 3%, 5%, and 10%) on the permeation parameters (flux, T_{lag}, and Q_{24}) for 5-FU is presented in Table 3. The oil at higher concentration was not considered in this study on account of its incomplete dissolution in the vehicle. The oil at different
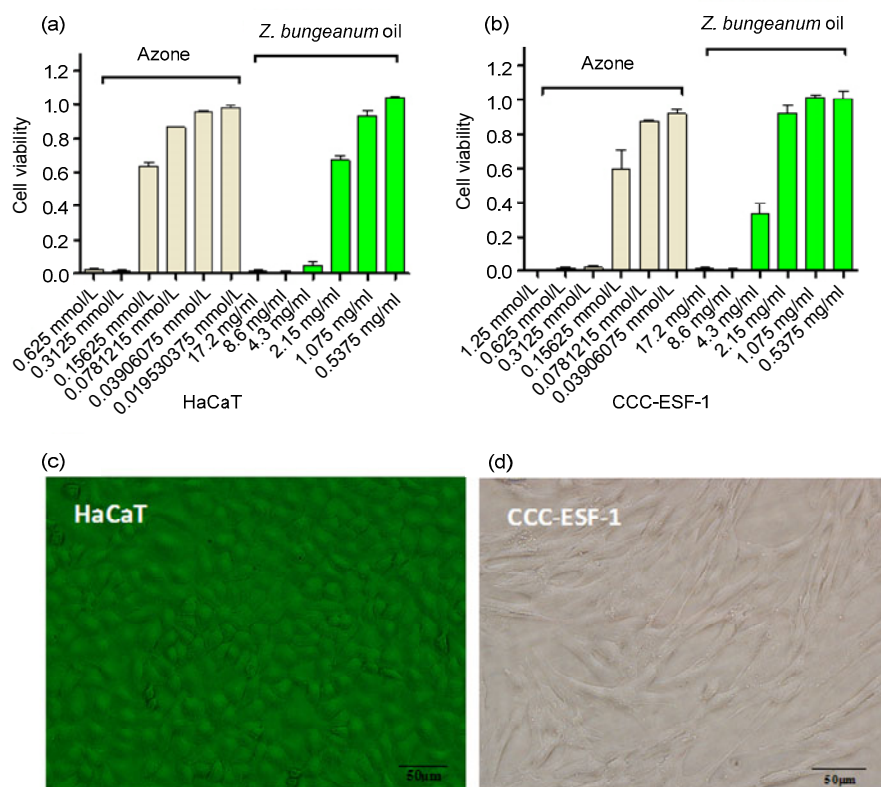
Fig. 1 Toxicities of _Zanthoxylum bungeanum_ oil and Azone in HaCaT keratinocyte and CCC-ESF-1 fibroblast cell lines
(a) Effects of selected permeation enhancers on HaCaT cell viability; (b) Effects of selected permeation enhancers on CCC-ESF-1 cell viability; (c) Cellular morphology of HaCaT; (d) Cellular morphology of CCC-ESF-1. Data in (a) and (b) are expressed as mean±SD (_n_=6)

Table 3 Percutaneous permeation parameters of 5-FU through excised rat skin

Penetration enhancer	Flux (μg/(cm²·h))	T_{lag} (h)	Q_{24} (μg/cm²)	ER	Solubility (mg/ml)
Control	1.16±0.21	2.04±0.83	49.77±8.85	1.0	4.93±0.40
0.2 mol/L Azone	217.96±20.62[*]	0.87±0.22[*]	7717.26±701.71[*]	187.8	7.60±0.46[*]
1% oil	7.45±1.19[*]	5.82±0.34[*]	323.24±51.78[*]	6.4	7.07±1.88[*]
3% oil	102.83±11.75[*]	6.48±0.83[*]	4949.27±377.64[*]	88.6	6.72±0.71[*]
5% oil	274.98±13.13[*]	5.61±0.79[*]	9450.17±443.77[*]	237.1	3.87±0.33[*]
10% oil	362.06±43.68[*]	2.85±0.69	11180.47±889.73[*]	312.1	3.58±0.54[*]

Each value represents the mean±SD (_n_=5), except for ER. [*] _P_<0.05, statistically significant difference between enhancers and control

concentrations exhibited statistically higher flux and Q_{24} values in comparison to those of the control, and showed a concentration-dependent manner as shown in Fig. 2. Meanwhile, apart from the 10% oil group, the T_{lag} value of the other oil groups was significantly superior to that of the control (_P_<0.05), and there was no significant difference between the control and the 10% oil group in terms of T_{lag} value. In addition, 0.2 mol/L Azone produced high permeation enhancement activity (ER=187.89) with low T_{lag} value compared with the control, which was consistent with the previous studies (Singh _et al._, 1993; He _et al._, 2008). However, the 5% oil group (ER= 237.05) exhibited better penetration enhancement activity than Azone, suggesting that _Z. bungeanum_ oil at a proper concentration could effectively promote the transdermal delivery of the hydrophilic drugs with a long T_{lag}.
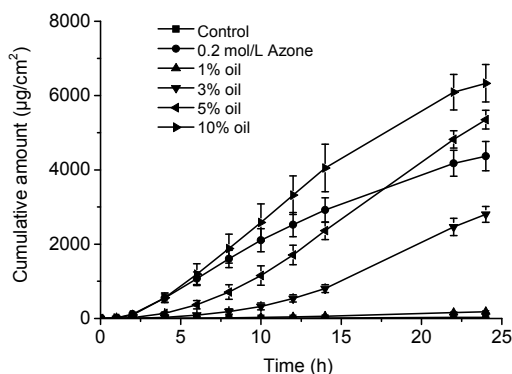
Fig. 2 Effects of *Zanthoxylum bungeanum* oil on percutaneous permeation of 5-FU through excised rat skin
Data are expressed as mean±SD (*n*=5)

The permeation profile of the cumulative amounts of IM penetrated across the rat skin is shown in Fig. 3. Similar to 5-FU, *Z. bungeanum* oil also markedly enhanced the percutaneous absorption of IM, whereas the T_{lag} value gradually decreased with increasing the oil concentration as presented in Table 4, implying that *Z. bungeanum* oil remarkably enhanced the penetration of the lipophilic drugs with a relative short T_{lag}.
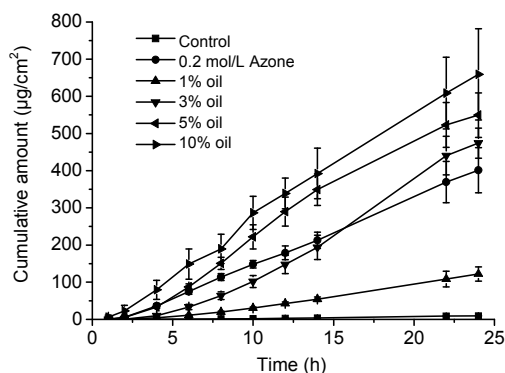

Fig. 3 Effects of *Zanthoxylum bungeanum* oil on percutaneous permeation of IM through excised rat skin
Data are expressed as mean±SD (*n*=5)

Based on the results of the penetration studies using two model drugs, it appears that *Z. bungeanum* oil could effectively facilitate the percutaneous absorptions of both hydrophilic and lipophilic drugs in a concentration-dependent manner, and also had a higher efficiency for the penetration of the hydrophilic drugs across the rat skin than for that of the lipophilic drugs according to the ER values. In addition, it is worth noting that the rat skin is more permeable than human skin due to the differences in their compositions and structure (Niazy, 1996), and more accurate evaluation should be measured using porcine skin or human skin (Reifenrath *et al.*, 1984).

3.4 Possible action mechanisms of *Z. bungeanum* oil

The mechanisms of action of transdermal penetration enhancers have been investigated by many research groups and possible explanations for the enhancement activity have been suggested as follows: (1) alteration of the thermodynamic activity of the drug causing that drug molecules readily penetrate across the skin (Song *et al.*, 2005; Zhang *et al.*, 2006); (2) increased partition of the drug into the SC (Vaddi *et al.*, 2002); (3) interactions with SC lipids or proteins resulting in disruption of the chemical structure or composition of the SC and disorganization of the highly ordered structures, thus increasing the delivery of the drug molecules (Williams and Barry, 2012). Certainly, other potential mechanisms for explaining the activity may also exist.

The results of the saturated solubilities and SC/vehicle partition coefficients of both 5-FU and IM after treatment with varying concentrations of *Z. bungeanum* oil are summarized in Tables 3, 4, and 5, respectively. For 5-FU, the saturated solubility slightly decreased with increasing the oil concentration, implying that the thermodynamic activity of 5-FU was altered to a certain extent in the presence of

Table 4 Percutaneous permeation parameters of IM through excised rat skin

Penetration enhancer	Flux (μg/(cm²·h))	T_{lag} (h)	Q_{24} (μg/cm²)	ER	Solubility (mg/ml)
Control	0.40±0.03	5.12±0.57	16.52±1.71	1.0	2.86±0.23
0.2 mol/L Azone	16.36±2.49[*]	0.66±0.22[*]	708.84±107.34[*]	40.9	2.85±0.29
1% oil	5.84±0.68[*]	4.70±0.23	215.74±33.58[*]	14.6	2.99±0.19
3% oil	21.80±3.77[*]	5.18±0.12	837.24±71.72[*]	54.5	3.69±0.04[*]
5% oil	28.78±3.54[*]	3.30±0.26[*]	978.42±87.42[*]	71.9	3.95±0.13[*]
10% oil	33.06±4.27[*]	1.89±0.60[*]	1164.10±216.50[*]	82.6	4.43±0.22[*]

Each value represents the mean±SD (*n*=5), except for ER. [*] *P*<0.05, statistically significant difference between enhancers and control

Table 5 Partition coefficients of model drugs treated with different enhancers (n=3)

Penetration enhancer	5-FU		IM	
	K	PER	K	PER
Control	0.109±0.014	−	0.170±0.020	−
1% oil	0.101±0.008	0.92	0.202±0.061[*]	1.19
3% oil	0.109±0.017	0.99	0.301±0.018[*]	1.77
5% oil	0.110±0.007	1.00	0.477±0.147[*]	2.80
10% oil	0.105±0.014	0.96	1.001±0.211[*]	5.86

K values are expressed as mean±SD (n=3). [*]P<0.05, statistically significant difference between enhancers and control. K: partition coefficient; PER: penetration enhancement ratio, and PER=(partition coefficient with essential oil treatment)/(partition coefficient without essential oil treatment)

the essential oil (Zhang et al., 2006). The saturated solubility of the 5-FU in the low-concentration oil (1% and 3%) was higher than that of the control, which was probably due to its moderate hydrophilicity. However, there was no significant difference between the control and the Z. bungeanum oil at different concentrations in terms of the SC/vehicle partition coefficient, which indicates that Z. bungeanum oil might contribute negligibly to the partitioning of hydrophilic drugs into SC due to its lipophilicity properties. Contrary to 5-FU, the SC/vehicle partition coefficient of IM gradually increased with the oil concentration increasing, and it was up to 5.85 times higher than that of the control when the oil concentration reached 10%. Furthermore, Z. bungeanum oil at different concentrations gradually improved the saturation solubility of IM, suggesting that the thermodynamic activity of IM was also significantly altered. Therefore, it implied that the Z. bungeanum oil could change the saturated solubility or the SC/vehicle partition coefficient of drugs to improve their delivery through the skin.

However, compared with the penetration ER, the alteration in the SC/vehicle partition coefficient or thermodynamic activity of two model drugs was relatively weak, suggesting that the Z. bungeanum oil probably enhanced the drug transdermal delivery substantially by changing the skin barrier property, but not altering the drug properties. Considering that T_{lag} is related to diffusivity, and it can reflect changes in both the length of the permeation path (h) and the diffusion coefficient (D) to suggest the interaction of the enhancer with the SC lipid and protein according to Eq. (2) as described above. As shown in Tables 3 and 4, for both of 5-FU and IM, the T_{lag} values were

roughly reduced with increasing the oil concentrations, indicating that the Z. bungeanum oil probably interacted with the SC lipids or protein to disrupt its barrier properties.

The FTIR absorption spectra were thus measured to investigate the biophysical changes of the rat skin SC properties due to its advantages on obtaining information about the lipids and keratin conformation in the SC. A representative spectrum of normal untreated rat skin SC shows that the major absorption peaks around 2850 and 2918 cm^{-1} due to symmetric and asymmetric C–H stretching vibration of the lipid alkyl chains (Zhang et al., 2007), the fatty acid carbonyl stretching peaks near 1733 cm^{-1} (Moore and Rerek, 2000; Jain et al., 2002), and two strong amide absorption peaks in the range of 1500–1700 cm^{-1}, i.e., 1652 cm^{-1} due to C=O stretching vibration of the amide I band and 1538 cm^{-1} due to the N–H bending vibration of the amide II band (Khurana et al., 2013). The shift to a higher frequency occurs when the CH$_2$ groups along the alkyl chain of lipids change from trans to gauche conformation, suggesting that the SC lipid is disturbed, and the peak areas of the two C–H absorption bands are proportional to the amount of the SC lipids (Zhang et al., 2007). The change in the amide I and amide II absorption peaks to a lower wavelength is observed to indicate the alterations of the keratin conformation under the effect of penetration enhancers (Zbytovská et al., 2004; He et al., 2009). In addition, the frequencies of the fatty acid carbonyl stretching mode, the keratin amide I and amide II modes are sensitive to hydrogen bonding, which can provide information on head-group interactions in the SC lipids (Moore and Rerek, 2000).

The FTIR absorption spectra of rat SC treated with varying concentrations of the Z. bungeanum oil are displayed in Fig. 4. Compared with untreated rat SC, FTIR of rat SC treated with the solvent showed no obvious shift in wavenumber or changes in peak area or peak height, suggesting that PG:water (80:20, v/v) system was inefficient to perturb or extract the SC lipids and proteins. The decrease in peak area of the C–H stretching bands and the shift in wavenumber were observed after treatment with different concentrations of Z. bungeanum oil, and the higher the oil concentration was, the greater the shift in C–H stretching vibration was (as presented in Fig. 5 and Table 6). This indicated that the Z. bungeanum oil
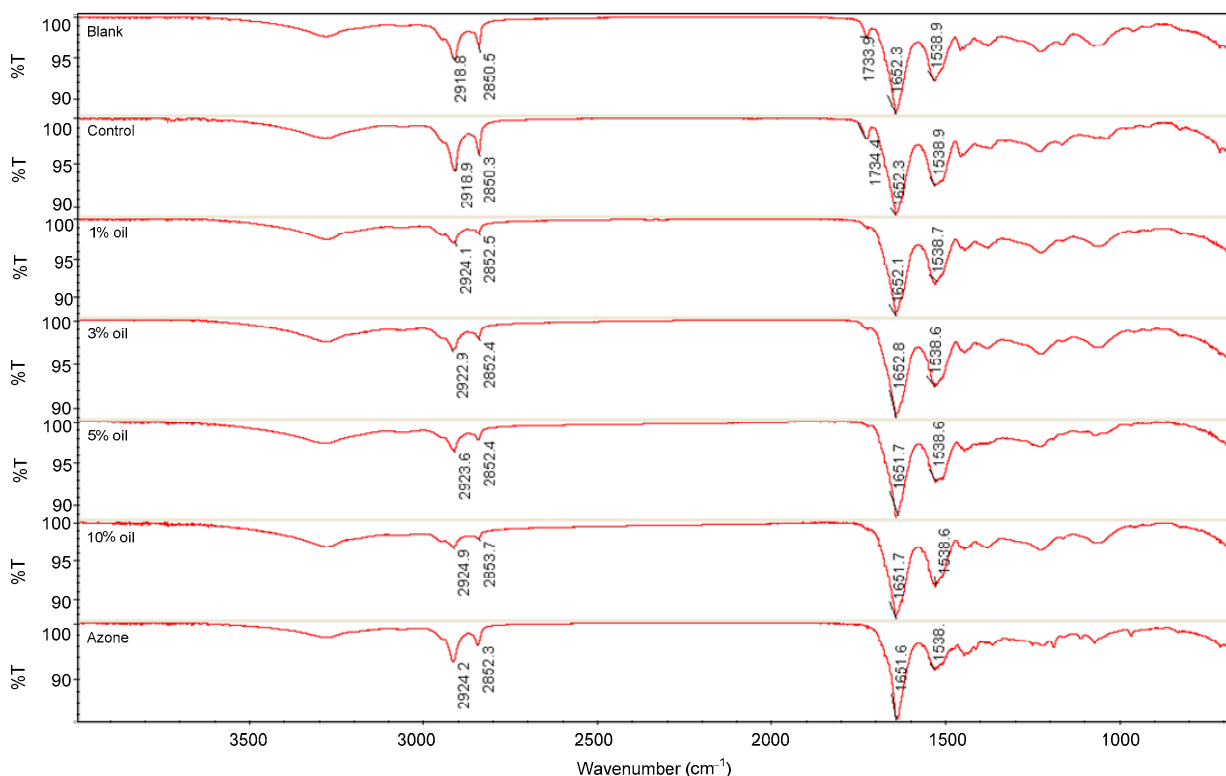
Fig. 4 FTIR spectra of the rat SC after treatment with different agents for 12 h

Table 6 Peak positions of lipids and amides of SC after treated with varying agents

Sample	Peak position of lipid (cm^{-1})		Peak position of amide (cm^{-1})	
	Asymmetric C–H stretching	Symmetric C–H stretching	I	II
Blank	2918.87	2850.45	1652.28	1538.98
Control	2918.92	2850.35	1652.25	1538.93
1% oil	2924.08	2852.57	1652.06	1538.71
3% oil	2922.99	2852.39	1651.88	1538.60
5% oil	2923.59	2852.38	1651.79	1538.60
10% oil	2924.97	2853.65	1651.79	1538.56
0.2 mol/L Azone	2924.23	2853.59	1651.70	1538.72

Fig. 5 Peak areas of C–H stretching of SC lipids treated with different penetration enhancers
Data are expressed as mean±SD (*n*=3). * Statistically significant difference between enhancers and control (*P*<0.05)

perturbed and extracted the SC lipids to alter the skin permeability. It was notable that the presence of 0.2 mol/L Azone only resulted in the shift of C–H stretching vibration to high wavenumber, whereas the peak areas of the two C–H stretching vibration absorption bands exhibited negligible change in comparison to those of untreated rat SC, which implied that 0.2 mol/L Azone increased the drug permeation mainly by perturbing the SC lipids. Meanwhile, the absence of –C=O peak of fatty acid indicated that the
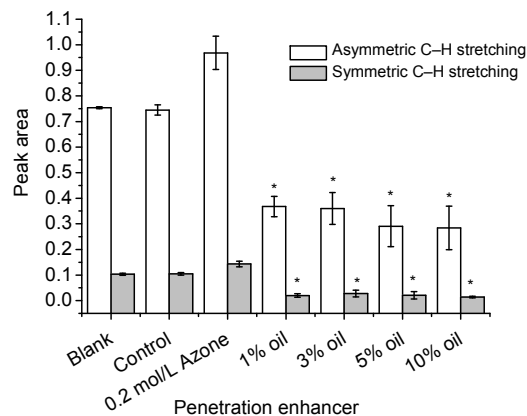
changes in the hydrogen bonding of the polar regions of the lipid bilayers might occur after treated with either *Z. bungeanum* oil or Azone. The wavelength movements of the amide I and amide II bands were narrow, perhaps indicating the *Z. bungeanum* oil contributed negligibly to the alteration of the keratin conformation.

4 Conclusions

Based on the results of this study, it was found that *Z. bungeanum* oil appeared to be a low skin toxicity in comparison to the well-established and standard penetration enhancer Azone, and effectively facilitated the percutaneous permeation of both 5-FU and IM through rat skin in a concentration-dependent manner. The partition studies exhibited that the partition coefficient for 5-FU was not altered, whereas it significantly increased for IM with incorporation of *Z. bungeanum* oil, indicating possible interaction of the oil with the SC lipids. Contrary to 5-FU, the saturated solubility of IM was gradually decreased with the increasing of the oil concentration, implying that the *Z. bungeanum* oil had altered the thermodynamic activities of both hydrophilic and lipophilic drugs to some extent. The results of FTIR studies showed that *Z. bungeanum* oil facilitated the drug permeation mainly by perturbing and extracting the SC lipids. In conclusion, *Z. bungeanum* oil could effectively facilitate the permeation of both hydrophilic and lipophilic drugs with low cytotoxicity.

Compliance with ethics guidelines

Yi LAN, Qing WU, Ying-qiu MAO, Qiong WANG, Jing AN, Yan-yan CHEN, Wen-ping WANG, Bo-chen ZHAO, Na LIU, and Ye-wen ZHANG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要：

本文题目：花椒挥发油作为天然经皮促透剂的细胞毒性及促透活性

Cytotoxicity and enhancement activity of essential oil from *Zanthoxylum bungeanum* Maxim. as a natural transdermal penetration enhancer

研究目的：研究花椒挥发油作为天然经皮促透剂的促透活性及其促透机制，同时评价其皮肤细胞毒性。

创新要点：首次评价了花椒挥发油的皮肤细胞毒性、经皮促透活性及其作用机制。

研究方法：利用表皮角质形成细胞（HaCaT）和真皮成纤维细胞（CCC-ESF-1）评价花椒挥发油的细胞毒性；采用亲水性及亲脂性模型药测定挥发油促透活性的基础上研究其促透作用机制。

重要结论：花椒挥发油具有良好促透活性并具有较低皮肤细胞毒性。

关键词组：花椒挥发油；天然经皮促透剂；透皮吸收；表皮角质形成细胞（HaCaT）；傅利叶变换红外光谱（ATR-FTIR）