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## Skin permeation profile and anti-inflammatory effect of anemonin extracted from weilingxian

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The aim of this study was to evaluate the skin permeability of anemonin, which was extracted from the Chinese herb weilingxian, and its potency of relieving the inflammation caused by rheumatoid arthritis (RA). To optimize the formulation, the solubility of anemonin in water and selected concentration of ethanol-water vehicles was determined. The effect of ethanol on the permeation of anemonin through human skin was then studied. Additionally, the influences of hydroxypropyl methylcellulose E50 (HPMC) and Carbomer 934 in different concentrations on the permeation of drug were investigated. Finally, the anti-inflammatory effect of the optimized formulation was assessed by murine model of xylene-induced ear edema. The results showed that the solubility and transdermal permeation of anemonin in ethanol-water vehicles linearly depended on the ethanol concentration. The combination of 30% ethanol and 3% Azone had a synergistic enhancement effect and was therefore selected for gel preparation. The 0.14% anemonin gel prepared with 1% HPMC exhibited the highest transdermal flux. The xylene-induced ear edema inhibitory rate of the optimized formulation was 48.85%. The results indicated that transdermal administration of anemonin is a potential modality for combating inflammation caused by RA.

### 1. Introduction

Rheumatoid arthritis (RA) is a long-term and chronic inflammatory autoimmune disease occurring frequently in older individuals, which is characterized by symmetric polyarticular inflammation and gradual joint destruction (Firestein 2003). Although they have not been shown to slow the progress of RA, oral NSAIDs remain the most commonly prescribed medications for the relief of RA-related pain and inflammation (Roelofs et al. 2008). Since oral NSAIDs pose a risk of adverse effects such as renal, hepatic, cardiac, and gastrointestinal toxicity, topical NSAIDs were developed (Doktor et al. 2013). Topical administration can provide a locally enhanced drug delivery for the superficial joint tissues by direct diffusion, however, few percutaneous NSAIDs have been demonstrated to show efficacy against joint inflammation in patients with RA (Xi et al. 2013; Kawai et al. 2010), the need for an efficient and safe alternative medication is substantial.

Herbal medicines have long been used to treat inflammatory diseases of the joints in Asian countries such as China, Korea, and Japan. Many *Clematis* species, for example, *Clematis mandshurica* Ruprecht (Hartog et al. 2008), *Clematis chinensis* Osbeck (Wu et al. 2010) and *Clematis vitalba* (Yesilada and Kupeli 2007) etc., were identified as having the potency to treat inflammation. Accumulative evidence has demonstrated that some *Clematis* species have chondroprotective effects *in vitro* and *in vivo* (Lee et al. 2007). The traditional Chinese herb weilingxian (WLX) (dried roots and rhizomes of *Clematis chinensis*

Osbeck, *Clematis hexapetala* Pall., and *Clematis mandshurica* Rupr.), with the functions of expelling wind-damp and activating meridians to stop pain, has long been used to treat rheumatic arthralgia, limbs numbness, tendons spasm, discomforts of flexion and extension (State Pharmacopoeia Committee of People's Republic of China 2010; Xiong et al. 2014).

It is well known that triterpenoid saponins are main anti-inflammatory active components of WLX (Xiong et al. 2014). More and more evidence has shown that anemonin, another major chemical constituent of WLX, also exhibits powerful anti-inflammatory activity. Anemonin, a dimer of protoanemonin (Fig. 1), can significantly reduce inflammatory response by inhibiting secretion of nitric oxide, endothelin-1, soluble intercellular adhesion molecule-1, interleukin-6, and thromboxane B<sub>2</sub> in lipopolysaccharid-induced rat intestinal microvascular endothelial and RAW 264.7 cells (Hu et al. 2009ab; Lee et al. 2008). Recently, it has been reported that anemonin appeared to possess the antiapoptosis ability and to protect mouse brain from ischemia and reperfusion damage (Jia et al. 2014).

As in some cases RA is restricted to only one or a few larger joints, percutaneous drug delivery provides an opportunity for drug localization at the target site. Meanwhile, the physico-chemical properties of anemonin (from Pubchem), molecular weight 192.16, melting point 157~158 °C, LogP 0.4, slightly soluble in cold water, soluble in hot ethanol and chloroform, the physical-chemical property of anemonin indicates it probably is a potential candidate for percutaneous application (Magnusson et al. 2004; Guy 2010).

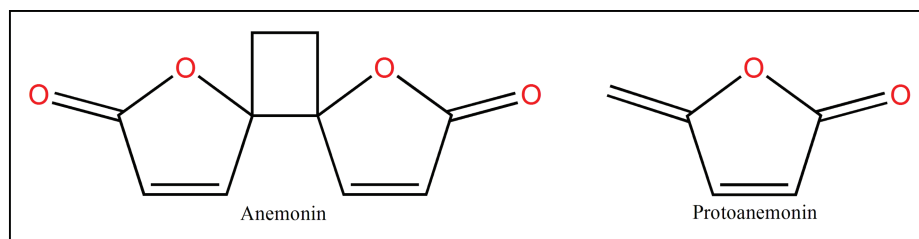


Fig. 1: Chemical structures of anemonin and protoanemonin.

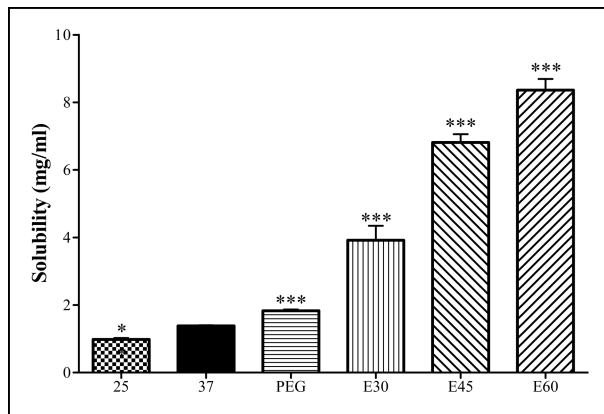


Fig. 2: Solubility of anemonin in different solvents. 25: aqueous solution at 25 °C; 37: aqueous solution at 37 °C; PEG: 20% PEG 400 in isotonic saline; E30: ethanol 30%; E45: ethanol 45%; E60: ethanol 60%. Values expressed as mean  $\pm$  SD (n=3). \*statistical difference compared with distilled water at 37 °C, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

For percutaneous delivery into deeper tissues for the treatment of RA, therapeutic agents must primarily pass through the outermost skin layer, stratum corneum (SC), epidermis, then dermis and subcutaneous fat. SC is only  $\sim 10\text{--}20\ \mu\text{m}$  thick across most parts of the human body, but provides a powerful barrier to the passive permeation of drugs (Prausnitz et al. 2004), therefore, the challenge remains to overcome the resistance of SC. The present study was designed to assess the permeability of anemonin through human skin and explore the potential for the treatment of inflammation. First, the solubility and permeability of anemonin in several solvents were determined. Subsequently, the effects of hydroxypropyl methylcellulose E50 (HPMC) and Carbomer 934 (CBR) on the permeation of anemonin were studied. At last, the formulation with the best performance was evaluated *in situ* in mice.

## 2. Investigations and results

### 2.1. Solubility

Figure 2 shows the results of solubility tests. The solubility of anemonin in water at 25 °C and 37 °C was  $0.982 \pm 0.07$  and  $1.38 \pm 0.04\ \text{mg/mL}$ , respectively. The values at 37 °C in 30 %, 45 %, 60 % (v/v) ethanol and isotonic saline containing 20 % (v/v) polyethylene glycol 400 (PEG 400) were  $3.92 \pm 0.08$ ,  $6.78 \pm 0.08$ ,  $8.36 \pm 0.11$  and  $1.83 \pm 0.06\ \text{mg/mL}$ , respectively.

### 2.2. In vitro skin permeation experiments

#### 2.2.1. Effect of ethanol water co-solvent on the permeation of anemonin

The results are shown in Fig. 3. The steady state flux value ( $J_{ss}$ ,  $\mu\text{g}/\text{cm}^2/\text{h}$ ) was:  $1.17 \pm 0.27$  for  $S_0$ ,  $9.36 \pm 0.50$  for  $S_1$ ,  $18.56 \pm 0.78$  for  $S_2$ ,  $1.08 \pm 0.17$  for  $S_3$ ,  $4.87 \pm 0.83$  for  $S_4$ ,  $7.30 \pm 0.29$  for  $S_5$  and  $13.77 \pm 0.20$  for  $S_6$ . 3% Azone ( $S_5$ ) significantly ( $P < 0.05$ ) increased the transdermal flux of anemonin compared to aqueous solution ( $S_0$ ) and the combination of 3% Azone (AZO) and 30% ethanol ( $S_6$ ) had a synergistic effect. 5% oleic acid ( $S_3$ ) achieved no increase in anemonin permeation, even when 5% oleic acid (OA) was combined with 30% ethanol ( $S_4$ ) the transdermal flux significantly ( $P < 0.05$ ) decreased with respect to 30% ethanol used alone ( $S_1$ ).

#### 2.2.2. Effect of gel polymer on the permeation of anemonin

As shown in Fig. 4 and Table 1. 1% HPMC (F4) demonstrated the best performance in penetration of anemonin, therefore it was selected as the base gel for the further investigations in which the effect of AZO was evaluated. Both 3% (F7) and 5% AZO (F8) significantly ( $P < 0.05$ ) enhanced the penetration of anemonin

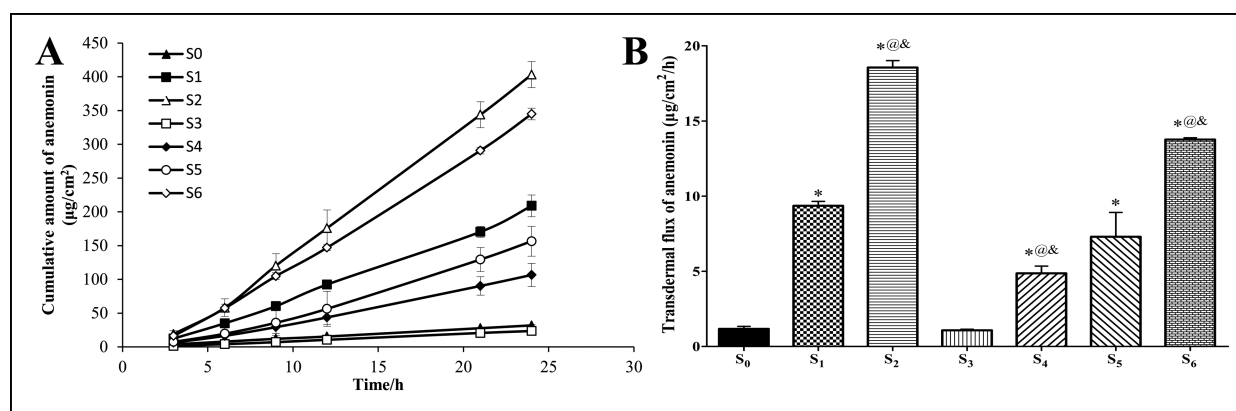


Fig. 3: A) In vitro permeation profiles versus time and B) transdermal fluxes of anemonin from solvents  $S_0$  to  $S_6$  through human skin (mean  $\pm$  SD, n=3). \* $P < 0.05$  statistical difference compared with  $S_0$ , @ $P < 0.05$  statistical difference compared with  $S_1$ , & $P < 0.05$  statistical difference compared with  $S_5$ , # $P < 0.05$  statistical difference compared with  $S_3$ .  $S_0$ : aqueous solution,  $S_1$ : 30% ethanol,  $S_2$ : 50% ethanol,  $S_3$ : 5% oleic acid (OA) + 4% tween20 (TW),  $S_4$ : 30% ethanol + 5% OA + 4% TW,  $S_5$ : 3% Azone + 4% TW,  $S_6$ : 30% ethanol + 3% Azone + 4% TW.

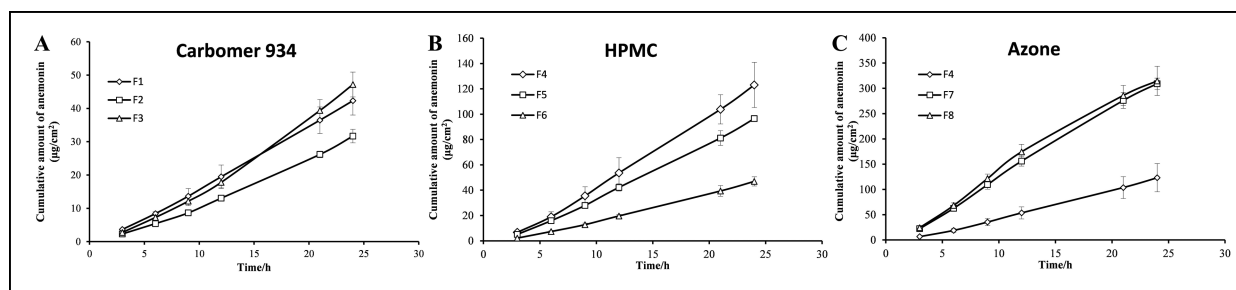


Fig. 4: In vitro permeation profiles of anemonin versus time from gels F1 to F8 through human skin. Anemonin permeation from different concentrations of A) Carbomer 934 and B) HPMC. C) Anemonin permeation from HPMC (1%) gels with different Azone concentrations. Values expressed as mean  $\pm$  SD (n = 3).

**Table 1: Profiles of anemonin gel delivering drug through human skin using a Franz diffusion cell *in vitro***

	$Q_{24h}$ ( $\mu\text{g}/\text{cm}^2$ )	$J_{ss}$ ( $\mu\text{g}/\text{cm}^2/\text{h}$ )	ER
REF	208.91 $\pm$ 16.24	9.361 $\pm$ 0.501	—
F1	42.31 $\pm$ 7.29*#	1.859 $\pm$ 0.214*#	—
F2	31.67 $\pm$ 2.05*#	1.415 $\pm$ 0.152*#	—
F3	47.18 $\pm$ 3.72*#	2.153 $\pm$ 0.181*#	—
F4	123.13 $\pm$ 17.83*	5.608 $\pm$ 0.692*	1
F5	96.54 $\pm$ 2.14*	4.377 $\pm$ 0.174*	—
F6	46.96 $\pm$ 3.65*#	2.151 $\pm$ 0.177*#	—
F7	309.34 $\pm$ 11.00*#	13.80 $\pm$ 0.47*#	2.46
F8	314.72 $\pm$ 28.78*#	13.91 $\pm$ 1.18*#	2.48

$Q_{24h}$ , cumulative amount of permeation by 24 h;  $J_{ss}$ , steady state flux; ER, enhancement ratio; REF, the reference solution without gel polymer. Values expressed as mean  $\pm$  SD (n = 3). Statistical difference compared with REF. \* $P < 0.05$ , with F4, # $P < 0.05$ .

compared to F4, but there was no statistical difference between them, so F7 was used for the following anti-inflammation study.

### 2.3. Effect of anemonin on xylene-induced ear edema in mice

As shown in Fig. 5, F7 (group C) and diclofenac gel (group D) significantly ( $P < 0.001$ ) reduced the edema with the inhibitory rate of 48.85 % and 55.42 % compared to the group treated with the saline (group A), respectively. The weight difference (mg) between the the left and right ear was: 13.43  $\pm$  2.37 for group A, 6.87  $\pm$  1.30 for group C and 5.99  $\pm$  1.11 for group D, respectively. The mice of group B only were applied with F7 but no ear edema was induced by xylene, the weight difference of the left and right ear was 0.47  $\pm$  0.17, what indicated that F7 almost did not cause irritation to mice ear.

### 3. Discussion

The present study was performed to explore the possibility of percutaneous delivery of anemonin from a gel formulation to resist topical inflammation associated with RA. The understanding of the effects of solvents on percutaneous drug absorption is important for transdermal preparations such as gel that is composed of volatile solvents which evaporate upon application on the skin (Intarakumhaeng and Li 2014). Ethanol is a solvent commonly used in transdermal formulations to increase the solubility of the drug in the vehicle. Ethanol can alter the SC barrier and the solubility properties of the tissue with a consequent improvement for drug partitioning into the membrane, and increase the thermodynamic activity of the solute when the solvent dissipates by skin absorption and evaporation (Lane 2013; Williams and Barry 2012). Anemonin is slightly soluble in water, therefore it is necessary to dissolve anemonin in a co-solvent in order to acquire appropriate solubility in formulations

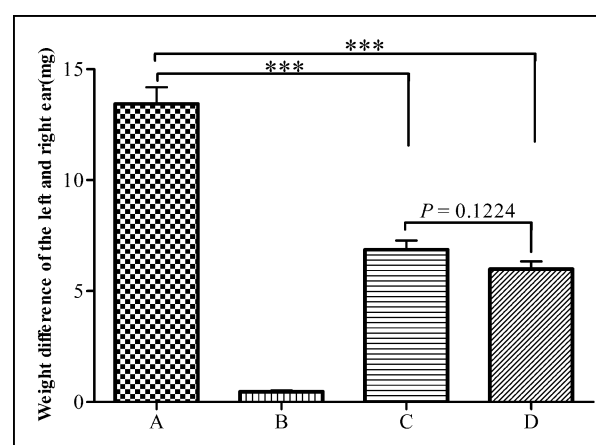


Fig. 5: Effect of anemonin on xylene-induced ear edema in mice. A: treated with saline gel and xylene; B: only treated with anemonin gel; C: treated with anemonin gel and xylene; D: treated with diclofenac gel and xylene. Values expressed as mean  $\pm$  SD (n = 10). \*statistical difference compared with A, \*\*\* $P < 0.001$ .

and finally enhance its permeation. As shown in Fig. 2, the solubility of anemonin in ethanol water co-solvent was significantly higher ( $P < 0.05$ ) than in water and increased with the increasing concentration of ethanol. As expected, the permeation of anemonin also increased with the increasing content of ethanol (Fig. 3). Considering the higher levels ethanol probably accompany a proportional increase in skin irritation, 30% ethanol-water (30:70, v/v) was selected as co-solvent in the present study. The gels have been proven to be a beneficial vehicle for topical drug delivery or for the localized drug action on skin such as in case of arthritis, osteoarthritis and degenerative joint disease (Sareen et al. 2011). RA is a symmetric polyarticular arthritis that primarily affects the small diarthrodial joints of the hands and feet (Firestein 2003), gel is more convenient to use and causes less obstacles to the joint activities in comparison with patches. HPMC and CBR are non-toxic and are approved for use in the field of medicine by FDA in drug delivery system. Thus, we selected gel as the vehicle in this study.

Various test parameters, such as the media composition and stirring speed, can have a huge impact on the outcome of *in vitro* diffusion studies with the vertical diffusion cell (Klein 2013). Based on previous work of our laboratory (Yu et al. 2013; Rao et al. 2013), isotonic saline containing 20% PEG 400 was selected as the receptor medium. The results of solubility experiment indicated the solubility of anemonin in isotonic saline containing 20% PEG 400 was significantly higher ( $P < 0.05$ ) than distilled water, it was enough to ensure the sink conditions during *in vitro* permeation experiments.

Although the genus *Clematis* has long been used to treat inflammatory diseases of the joints, fresh *Clematis* is hardly employed. The reason is that the entire genus *Clematis* contains protoanemonin which is extremely irritating to the

**Table 2: Compositions of gel formulations (% w/w)**

Materials	F1	F2	F3	F4	F5	F6	F7	F8
Anemonin	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Carbomer 934	1.00	3.00	5.00	–	–	–	–	–
HPMC	–	–	–	1.00	3.00	5.00	1.00	1.00
Triethanolamine	1.35	4.05	6.75	–	–	–	–	–
Azone	–	–	–	–	–	–	3.00	5.00
Tween 20	–	–	–	–	–	–	4.00	4.00
Ethanol	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Distilled water	100	100	100	100	100	100	100	100
q.s. to make								

skin and mucous membranes, protoanemonin can rapidly dimerize with itself to form anemonin in the drying process of plants (Chawla et al. 2012). But even the dried *Clematis* still contain protoanemonin to some extent. Extracting anemonin from dry WLX avoids the adverse effects of protoanemonin. Anti-inflammatory experiments demonstrated that anemonin gel caused no irritation to mice ear and efficiently inhibited the xylene-induced ear edema by 48.85%. It indicated that administration of anemonin by a transdermal dosage from to treat inflammation is effective and safe.

The results of *in vitro* and *in situ* experiments showed that it is possible to deliver anemonin through skin to treat the inflammation caused by RA. The final formulation contained 30% ethanol, 3% AZO and 4% TW, this composition is typically used (Williams and Barry 2012; Lane 2013). The *in situ* experiments demonstrated that the formulation caused no apparent irritation to mice ear. This may indicate that the composition is suitable for further development of a transdermal formulation. It will be necessary to investigate whether anemonin can be delivered into synovial fluids *in vivo*. In order to ensure an appropriate concentration of anemonin in synovial fluids additional investigation may also be undertaken to further modify the formulation so as to transport as much as possible of anemonin from skin to deeper sites.

## 4. Experimental

### 4.1. Materials

Anemonin (extracted from WLX by our laboratory, purity  $\geq 95\%$ ). The anemonin reference substance (Lot numbers: 111768–200505, purity  $\geq 99\%$ ) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Diclofenac sodium (purity  $\geq 99\%$ ) used as a positive control was purchased from Aladdin Industrial Corporation (Shanghai, China). HPMC was purchased from Colorcon Coating Technology Co. Ltd (Shanghai, China). CBR was manufactured by BF Goodrich Company (USA). Other materials used in this study were purchased from Huadong Medicine Co. Ltd (Hangzhou, China) and were of analytical or high performance liquid chromatography (HPLC) gradient grade.

### 4.2. Human skin and animals

Human abdominal skin was supplied by the Anatomy Department of Zhejiang University and has been approved by Experimental Animal Use Committee of Zhejiang University, China.

Male Kunming mice, 5–6 weeks old, weighing 25–30 g were obtained from the Experimental Animal Center of Zhejiang University (Hangzhou, China). The animals were maintained in collective cages at  $22 \pm 2^\circ\text{C}$  and relative humidity between 40% and 70% under a 12 h light/dark cycle. The animals had free access to food and water. Animal care and experiments were conducted in accordance with the Guidelines of Zhejiang University Animal Care Committee.

### 4.3. Solubility measurement

An excess of anemonin reference substance was placed in contact with 1 mL of solvent in a sealed glass tube. The tube was maintained in a constant temperature incubator shaker at  $37^\circ\text{C}$  for 72 h to produce a saturated solution

with visible excess chemical. The saturated solution was centrifuged and the supernatant was filtered through a  $0.45\ \mu\text{m}$  polytetrafluoroethylene membrane. The concentration of drug in the saturated solution was quantified by HPLC after appropriate dilution with the mobile phase.

### 4.4. Preparation of co-solvent solution of anemonin

To determine the effect of ethanol on the permeation of anemonin, the tested formulations were prepared by adding anemonin into several different combinations of solvents as follows: 30% ethanol (v/v, S<sub>1</sub>), 50% ethanol (v/v, S<sub>2</sub>), 5% OA + 4% Tween 20 (TW) (v/v, S<sub>3</sub>), 30% ethanol + 5% OA + 4% TW (v/v, S<sub>4</sub>), 3% AZO + 4% TW (v/v, S<sub>5</sub>), 30% ethanol + 3% AZO + 4% TW (v/v, S<sub>6</sub>), then distilled water was added to adjust to the total volume as prescribed and stirred overnight. Anemonin aqueous solution (S<sub>0</sub>) was used as the control solution. The concentration of anemonin in all solutions was 1.4 mg/mL. The addition of TW served as a wetting agent in the test solution of water to reduce the surface tension of water, increasing the solubility of AZO and OA in water.

### 4.5. Preparation of gel formulations

To evaluate the influence of gel polymer on the anemonin permeation, HPMC and CBR were selected to be used in tested formulations. Briefly, the appropriate HPMC/CBR was slowly dispersed in distilled water. Drug was dissolved in ethanol and added dropwise into the gel to be completely mixed. Optionally a chemical enhancer was added into the gel as prescribed and the anemonin concentration in all formulations was 0.14% (w/w). The CBR gel was neutralized with triethanolamine as prescribed. Table 2 reports the exact composition of the gels.

### 4.6. *In vitro* skin permeation studies

As described previously (Rao et al. 2013; Yu et al. 2013), the frozen human cadaver skin without adhering subcutaneous fatty tissue was thawed at ambient temperature, and then washed gently three times with saline prior to use. The skin was mounted onto the modified Franz diffusion cells (TK-6A, Kai kai technology trading company, Shanghai, China) with the SC side facing the donor compartment. The interface area was  $2.8\ \text{cm}^2$ , and the receptor volume was 6.8 mL. The diffusion cell was filled with isotonic saline for transport kinetic balance of skin. After a one hour equilibration period, the isotonic saline was abandoned and the receptor cell was refilled with degassed isotonic saline containing 20% PEG 400 as solubilizer and 1 g formulation was applied on each donor chamber of Franz cells. The receptor compartment was sampled at predetermined time intervals, 1 mL was collected and replaced by fresh receptor medium. The receptor cell was maintained at  $37 \pm 0.5^\circ\text{C}$  and stirred at a constant rate of 300 rpm by a Teflon-coated magnetic bar to keep them well mixed. The permeation experiments were carried out for 24 h.

### 4.7. Anti-inflammatory effect assessment

The experiment was carried out as described previously (Wu et al. 2006; Patel et al. 2009) with some modifications. Briefly, the mice were divided into four groups, each comprising 10 animals. Group A received 0.03 ml normal saline and served as vehicle control, group B and C were treated with 0.03 ml F7 on the anterior and posterior surfaces of the right ear evenly, respectively, and group D received 0.03 ml diclofenac gel (the composition similar to F7 besides anemonin replaced by diclofenac sodium) as a positive control. Thirty minutes after administration of drug, the gels remaining on the skin surface of group C and D were removed by gentle washing with saline. Subsequently, apart from the group B, all the mice in other groups were applied with 0.03 ml xylene on the anterior and posterior surfaces of the right ear evenly. After 2 h of edema induction, the mice were sacrificed

and 9 mm circular sections were cut from the right and the left ear by the puncher. Inflammation was measured as edema formation and quantified by the weight difference between the right and the left ear samples.

#### 4.8. RP-HPLC analysis

A rapid and sensitive RP-HPLC method was developed to simultaneously measure anemonin concentrations in the receptor medium. Samples were analyzed at a temperature of 35 °C using an Agilent 1100 series automated HPLC system. The instrument was fitted with an Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm, Agilent). Detection wavelength was set at 220 nm. The injection volume was 20 μL. An isocratic elution program was used with the mobile phase of acetonitrile:phosphate-buffer (75:25, v/v, pH = 3.0 with H<sub>3</sub>PO<sub>4</sub>) and a flow rate of 1 mL/min. The amount of drug permeated per square centimeter of skin was calculated from the standard curve. The retention time for anemonin was approximately 8.5 min. The method provided good precision and linearity in the required concentration range (0.4–150 μg/ml, R<sup>2</sup> = 0.999).

Cumulative amount ( $Q_{24h}$ , μg/cm<sup>2</sup>) of anemonin collected in the receiver was plotted as a function of time. The flux value ( $J_{ss}$ ) for each experiment was obtained from the slope of the linear portion of the data fitted by regression analysis. Penetration-enhancing activities were expressed as enhancement ratios (ER), i.e., the ratio of the flux value with enhancer to that obtained without enhancer (Jantharaprapap and Stagni 2007).

#### 4.9. Statistical analysis

All skin permeability experiments were repeated at least three times and values of the measured index were expressed as the mean ± standard deviation (SD). Mean, SD, scatterplots, histograms and linear regression analyses were calculated using Microsoft Excel 2010. Analysis of variance (ANOVA), Tukey *post hoc* test, and Student *t*-test were performed with SPSS 20.0 for Windows (v 20.0; SPSS, Inc., Chicago, IL, USA). A value of  $P < 0.05$  was considered as the statistical difference.

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