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The Effect of Clove Oil on the Transdermal Delivery of Ibuprofen in the Rabbit by In Vitro and In Vivo Methods

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The study was designed to evaluate skin permeation enhancement effect of essential oils from *Eugenia caryophyllata* (clove oil) in rabbits and to compare the in vitro absorption and in vivo permeation using ibuprofen as a model drug. The in vitro results indicated a significant permeation enhancement effect of the clove oil. The group with 1% oil appeared to the flux (239 μg/cm^2/hr), and 3% oil was 293 μg/cm^2/hr to some extent similar with 2% azone group (327 μg/cm^2/hr). The enhancement ratio of clove oil was 7.3. In vivo results also demonstrated that clove oil showed a significant permeation enhancement effect, but the enhancement of clove oil was relatively weak than in vitro. The group with 3% oil exhibited the higher value of area under the curve (AUC) of 80.8 μg/mL·hr, which was 2.4 times the high of control. The AUC value of 3% oil group was similar to that of 2% azone group (89.8 μg/mL·hr). The GC-MS results indicated eugenol and acetyleugenol identified from clove oil might mainly contribute to enhance in vitro and in vivo absorption of ibuprofen because of its large quantities (90.93%).

Keywords skin permeation enhancement; clove oil; ibuprofen; penetration enhancer; rabbit

INTRODUCTION

The transdermal drug delivery system offers many advantages over the conventional dosage forms, such as improved patient compliance (Brown & Langer, 1988), reduced side effects, no hepatic first pass effect, and convenience on interruption or termination of treatment when necessary (Godwin & Michniak, 1999; Kydonieus, 1987). Moreover, such a non-invasive drug delivery route, compared with the oral administration, significantly reduces drug degradation due to its lower metabolic activity at the site of administration (Roy, 1999) as well as the fact that the hepatic circulation, a major site of potential drug metabolism, is bypassed (Barry, 1983; Wester & Maibach, 1992).

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Despite the important advantages of transdermal drug delivery, many drugs are often precluded because of the stratum corneum barrier, the thin outermost layer of the skin, which is comprised of a regular array of protein-rich cells that provides the rate-limiting step for drug transport. One approach is the co-administration of chemical enhancers, which increases the solubility of the drug in stratum corneum or disrupts the lipid matrix of stratum corneum or interacts with the intracellular protein. In this way, many compounds were developed and later evaluated for their percutaneous drug transport enhancing activity. Some plant essential oils, such as terpenes and other compounds; showing good penetration enhancing effects on various drugs (Gao & Singh, 1998a; Zhang, Hu, Li, Gao, Zhu, & Su, 2006), appear to be promising candidates for clinically acceptable enhancers (Williams & Barry, 1991). They were reported to have good toxicological profiles, high enhancement abilities of percutaneous penetration, and low skin irritation at low concentrations (1–5%) (Kommuru, Khan, & Reddy, 1998; Kunta, Goskonda, & Brotherton, 1997). A variety of volatile oils have been shown to increase the percutaneous absorption of both hydrophilic and lipophilic drugs (Gao & Singh, 1998b).

Ibuprofen, a non-steroidal anti-inflammatory drug (NASAID) is very effective for the systemic treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis.

Ibuprofen was formulated into many topical preparations to reduce the adverse side effects and avoid the hepatic first-pass metabolism. But it is difficult to maintain effective concentrations by topical delivery of ibuprofen due to its poor skin permeation ability (Yano, Nakagawa, Tsuji, & Noda, 1986). In order to enhance the permeation of ibuprofen, many methods have been used (Chen, Chang, Du, Li, Xu, & Yang, 2006; Heard, Gallagher, Harwood, & Maguire, 2003; Park, Chang, Hahn, & Chi, 2000; Stott, Williams, & Barry, 1998;).

In this study, we selected clove oil to investigate the effects on the *in vitro* and in vivo percutaneous absorption of ibuprofen. We first investigated the in vitro skin permeation enhancement effect of clove oil formulated in Carbopol gel in

rabbit skin. The in vivo experiments with rabbit were also performed.

MATERIALS AND METHODS

Chemicals

Ibuprofen was purchased from Xinhua Pharmaceutical Ltd. (Shandong, China). *Eugenia caryophyllata* was supplied from traditional Chinese Medicine Store (Shanghai, China). Azone was obtained from Shanghai Chemical Company (Purity, 99.3%, Shanghai, China). Carbopol 940 was a gift sample from BF Goodrich Company (USA). Other chemicals and solvents were of analytical grade.

Animals Male New Zealand white rabbits weighing 2.2 ± 0.2 kg were obtained from the Animal Center of Shanghai Jiao Tong University (Shanghai, China). The studies examined in this article have been carried out in accordance with the guidelines of the animal ethics committee at Shanghai Jiao Tong University, China.

Supercritical Fluid Extraction The dry *Eugenia caryophyllata* was pulverized. A 1 kg amount of the pulverized sample was packed into a 2 L sample cartridge. Supercritical fluid extraction was performed on a HL-40a supercritical fluid extractor (HuaLi SFE Equipment Ltd, China). The extraction temperature was set at 40 ± 2°C. Liquid carbon dioxide at pressure 10 ± 0.2 MPa was then allowed to flow into the sample cartridge. The extraction process was run for 2 hr. Clove oil was obtained by reducing pressure of supercritical carbon dioxide. The clove oil was prepared three batch. The composition of the clove oil was investigated use GC-MS.

Gas Chromatography-Mass Spectrometry Analysis

The essential oil was analyzed with GC-MS using a Hewlett-Packard (HP) 6890 series gas chromatograph interfaced to an HP 5973 mass-selective detector (MSD). An HP-mass ChemStation Data system was used for identifying the components.

Clove oil of 1 μL was injected manually into a HP-5 MS capillary column (30m × 0.25 mm, 0.32 μm, i.d.) using the following temperature program. The initial temperature was 80°C for 3 min, and then increased to 250°C, hold 3 min. The injection temperature was 250°C, the MS source temperature was 250°C. The percentage composition of the essential oils was computed from the GC peak areas. Chromatographic peaks were checked for homogeneity with the aid of mass chromatograms with characteristic ion fragments. The Mass Spectra Library database was used for automatic identification of the peaks.

Preparation of Gel The gel was composed of ibuprofen (2% w/v), Carbopol 940 (2%), ethanol (10%), azone (2%) and triethanolamine (3%). Carbopol powder was dispersed into the water phase and set for 24 hr at room temperature, followed by adding triethanolamine for neutralization to form a homogeneous gel. Ibuprofen mixed with clove oil or azone if necessary was dissolved in ethanol. The alcoholic solution was slowly added to the vortex of agitated gel. The remaining water phase was added to the gel with continuous stirring.

In Vitro Skin Permeation Experiments

The abdominal skin of rabbit was used in the experiment. After the rabbits were sacrificed by spinal dislocation, the abdominal skin was carefully removed leaving the fat tissue behind. The skin was examined under magnifying lens for damage or diseased conditions, and any skin with the disrupted barrier was excluded in this study. Freshly prepared abdominal rabbit skin was used in the diffusion experiments. Integrity of each piece of prepared skin was ensured (i.e., epidermis with stratum corneum and dermis). Permeation experiments were carried out using Modified Franz vertical diffusion cell systems. The donor cell was filled with 1.0 g of test gel and occluded with aluminum foil. The receiver solution was 5.0 ml of phosphate-buffered saline at pH 7.4 (PBS). The effective surface area available for permeation was 0.78 cm^2. The excised skin was mounted between the donor and receptor cells with epidermal side facing the donor cell. The temperature of the cells and the contents was kept constant at 37 ± 0.5°C throughout each experiment. The content of the receiver compartment was stirred with a magnetic bar at 100 rpm. All of the receiver solution was withdrawn at predetermined time points,and replaced with an equivalent volume of phosphate buffered saline (0.2 mL) to maintained the constant volume. Control experiments were also performed without enhancers. All the experiments were run for 12 hr. The samples were assayed using HPLC.

In Vivo Percutaneous Absorption

The rabbits were housed individually over 2 weeks in a temperature-controlled (25 ± 1°C) and relative humidity-controlled (50–60%) environment and had free access to a standard diet and water 1 week prior to the experiments. The rabbits were fasted for 24 hr and then whose dorsal regions were carefully depilated by depilatory cream and the skins were cleaned by wiping with water containing cotton under pentobarbital anesthesia. The rabbits were randomly divided into three groups of five each: the control (no enhancer), two of clove oil (3%) and 2% azone. The gel was applied to 8 × 8 cm^2 area of the depilated dorsal area of the rabbit, covered with a non-porous silicone membrane and wrapped with an elastic adhensive bandage. The amount of gel used was equivalent to a dose of 50 mg/kg ibuprofen. A 0.25 mL of blood sample was withdrawn from the central ear artery at predetermined times over 12 hr. The blood samples were centrifuged at 10,000 rpm for 10 min to obtain 0.1 mL of the plasma.

HPLC Determination of Ibuprofen

Ibuprofen was analyzed by reversed phase HPLC system containing 5 μm Crosmosil (4.6 mm × 25 cm) particles in an analytical column from Nacalai Tesque, a Shimadzu LC-10 pump system, a Shimadu LC-10 autoinjector, a Shimadzu LC-10 detector and a Shimadzu CR-6A integrator. The mobile phase was an acetonitrile–sodium acetate buffer solution (pH 2.5) (70:30) (v/v) mixture. The flow rate was 1.0 $mLmin^{-1}$. and the detection wavelength was set at 264 nm.

Phosphate buffer (0.1 mL, pH 7.4), water (0.5 mL) and ethyl acetate (6 mL) were added to 0.1 mL of the plasma samples. It was then mixed for 10 min using the rotamix and centrifuged at 3000 rpm for 5 min. The organic layer (6 mL) were transferred to a clean test tube and evaporated in a centrifugal evaporator at 40°C, the residue was then dissolved in a solution (acetonitrile: sodium acetate = 7:3), centrifuged at 10000 rpm for 5 min, and the solution (20 μL) was injected into the HPLC system.

Skin Irritation Test and Histological Evaluation in Rabbit (Lu, Lee, & Rao, 1992)

Skin Irritation. Rabbits were used to evaluate the effect on skin irritation. Each individual formulation was tested in four rabbits. The patch was applied to the rabbit for 24, 48, and 72 hr and then the skin surface was visually examined for redness, discoloration and swell. 24 hours before the test, rabbit were shaved at the abdominal site using a clipper. After gel application, all rabbits were housed in stainless-steel cages equipped with feeders and automatic water dispensers.

H & E Staining (Motlekar, Srivenugopal, Wachtel, & Younan, 2006). Formulations containing 3% clove oil were administered to rabbit as described above. The skin tissues before administration of formulation were prepared as control samples. At the end of the in vivo experiment, the skin tissues were isolated from the rabbits and fixed in formalin for processing. The tissue specimens were embedded in paraffin and cut into sections. The sections were stained with haematoxylin and eosin (H&E) and examined under an optical microscope.

Data Analysis and Statistics

The cumulative amount of Ibuprofen permeated per unit skin surface area was plotted against time, and slope of the linear portion of the plot was estimated as steady state flux (J_{ss}). The permeability coefficient (K_p) was calculated as described by Scheuplein (1978).

$$K_p = J_{ss} / C_v$$

where C_v is the total donor concentration of the Ibuprofen. The enhancement ratio (ER) was calculated as described by Williams and Barry (1991).

$ER = K_p$ after application of penetration enhancer/K_p before application of penetration enhancer.

Results are expressed as the mean of *SE* of at least three experiments. Statistical significance was assessed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance.

RESULTS

In Vitro Skin Permeation

The effect of volatile oils on the in vitro percutaneous absorption profiles of ibuprofen through the rabbit skin is show in Figure 1. Figure shows that the permeation of ibuprofen through excised rabbit skin followed zero order release kinetics and accorded with the Fick's first diffusion law. The ibuprofen flux of 3% oil group was 293 $\mu g/cm^2/hr$, which was significantly higher ($p < 0.05$) than the control of 40 $\mu g/cm^2/hr$.

The ibuprofen flux of 2% azone group was 327 $\mu g/cm^2/hr$, which was slightly large than clove oil group at the same concentration.

The effect of clove oil on the flux of ibuprofen is listed in Table1. At 1% and 3% concentrations, clove oil exerted a significant enhancement to the flux of ibuprofen through excised rabbit skin. As shown in Table 1, at 1% and 3% oil concentration, the ER (enhancement ratio) of ibuprofen was 6.0 and 7.3 times, which was higher than that of the control.

In Vivo Percutaneous Absorption

Azone was used as a positive control for clove oil to accelerate percutaneous absorption of ibuprofen and its concentration in gel was fixed at 2%. The plasma concentrations of ibuprofen after the transdermal administration of gels with clove oil at 3% concentrations to rabbits are shown in Figure 2. Some pharmacokinetic parameters are summarized in Table 2. Clove oil showed a significant permeation enhancement to transdermal delivery of ibuprofen through skin barrier. The group with 3% oil exhibited the AUC value of 80.8 μg/mL·hr,

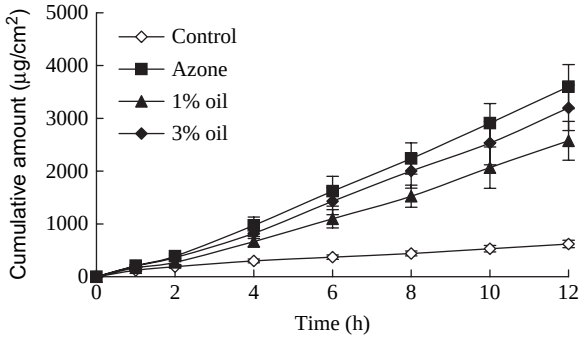

FIGURE 1. Effect of clove oil on the permeation of ibuprofen through excised rabbit skin. results are expressed as the $M \pm SE$ of at least three experiments.

TABLE 1
Effect of Clove Oil on the Flux of Ibuprofen Through Excised Rabbit Skin

Formula	Flux (μg/cm^2/hr)	ER
Control	40.0 ± 6.5	
1% clove oil	239 ± 30.2*	6.0
3% clove oil	293 ± 46.3*	7.3
2% Azone	327 ± 50.7*	8.2

Each value represents the $M \pm SE$ of at least three experiments.

NS: not significantly different, *$p < 0.05$, compared with control, **$p < 0.01$, compared with control.

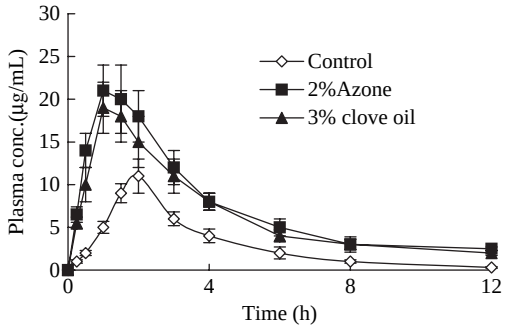

FIGURE 2. Plasma concentration profile of ibuprofen after the transdermal administration of carbopol hydrogel to rabbits at a dose of 50 mg/kg. results are expressed as the $M \pm SE$ of at least three experiments.

which was 2.4 times the high of control (33.2 μg/mL·hr). The *AUC* value of 3% oil group was similar to that of 2% azone group (89.8 μg/mL·hr). Clove oil appeared a higher permeation enhancement compared with the control. As listed in Table 2, 3% oil group exhibited the C_{max} value (19.5 μg/mL) and the T_{max} value (0.9 hr). The effects of group with 3% oil were similar to those of 2% azone group in C_{max} value (22.7 μg/mL) and T_{max} value (1.0 hr).

TABLE 2
Pharmacokinetic Parameters of Ibuprofen in Rabbits After the Transdermal Administration of Carbopol Hydrogel to Rabbits at a Dose of 50 mg/kg

	C_{max} (μg/mL)	T_{max} (hr)	AUC_{0-12} (μg/mL•hr)
Control	11 ± 2.1	2.3 ± 0.29	33.2 ± 4.3
3% clove oil	22 ± 3.5	0.9 ± 0.15	80.8 ± 11.7*
2% azone	19 ± 3.3	1.0 ± 0.19	89.8 ± 12.5*

Each value represents the $M \pm SE$ of at least three experiments. NS: not significantly different; *$p < 0.05$, compared with control, **$p < 0.01$, compared with control.

Determination of the Major Constituents from Clove Oil

The principal compounds were identified in GC analysis by means of the mass spectral fragmentation patterns. The profiles of the clove oils extracted from *Eugenia caryophyllata* are summarized in Table 3. The major components from clove oils were terpenes with various carbon numbers. Eleven of them were identified according to the mass spectrum of each constituent. By comparing the mass spectra data, peaks 1, 2, 3, 4, 5, 6 were identified as 3-carene, eucalyptol, phenol, 2-methoxy, phenol, 4-(2-propenyl), eugenol, phenol, 2-methoxy-4-propyl. Among of them, eugenol relative content was 82.65%. Peaks 7, 8, 9, 10, 11were identified as vanillin, caryophyllene oxide, α-caryophyllene, acetyleugenol, and santalol. The compound with the highest content in clove oil was eugenol, the second content is acetyleugenol.

Histological Evaluation of Skin Tissues

A major concern regarding skin absorption enhancers is their potential to cause irritation. To address this concern, the effect of clove oil on skin tissue was examined using H&E staining. For the formulation used in the studies, the dose of clove oil was 3% (w/v). As show in Figure 3, the morphology of the skin tissues, including congestion of skin capillary, was not visibly affected by the clove oil. In addition, no redness, discoloration and swell were detected in the clove oil treated group.

DISCUSSION

The efficacy of clove oil was systemically assessed using in vitro and in vivo methods. The compounds of clove oil were terpenes with various carbon numbers. The oil effectively accelerated ibuprofen permeation across the skin. The permeation enhancement of clove oil was similar to that of azone. The commonly used enhancers, azone were used to promote skin permeation of ibuprofen for comparison with clove oil. The concentration of the enhancers in hydrogels was set at 2% since this concentration produced effective enhancement on skin permeability in some investigations (Zhao & Singh, 1998). The flux of ibuprofen did not significantly differ ($p > 0.05$) between the azone and clove oil, many of the publications on enhancer mechanisms suggest that they enter the skin lipids and disruption of the highly ordered structure of struatum corneum lipids. A diffusing penetrant therefore experiences a more fluid environment and permeates more rapidly (Barry, 1991; Yamane, Williams, & Barry, 1995). The clove oil gave the good absorption profiles. These results may suggest that clove oil act in combination to help the partitioning of the drug into the skin or its penetration through the skin.

TABLE 3
Identification of Compounds from Clove Oil Under Supercritical Fluid Extraction in GC-MS

Peak no.	Retention Time (min)	Molecular Weight	Compound	Relative Content (%)
1	4.39	136	3-carene	0.01
2	6.64	154	eucalyptol	0.04
3	13.02	128	phenol,2-methoxy	0.05
4	16.36	134	phenol,4-(2-propenyl)	0.12
5	19.24	164	eugenol	82.65
6	21.02	170	phenol,2-methoxy-4-propyl	0.11
7	21.63	152	vanillin	0.33
8	22.53	220	caryophyllene oxide	0.03
9	23.25	204	α-caryophyllene	0.09
10	26.08	206	acetyleugenol	8.28
11	27.81	230	santalol	0.06

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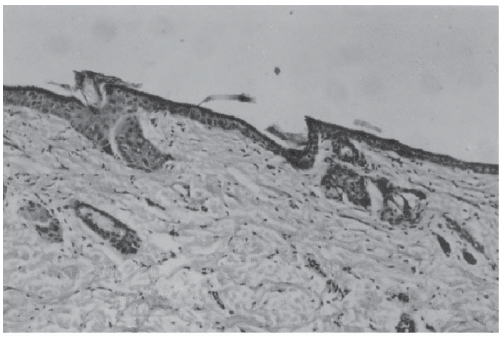

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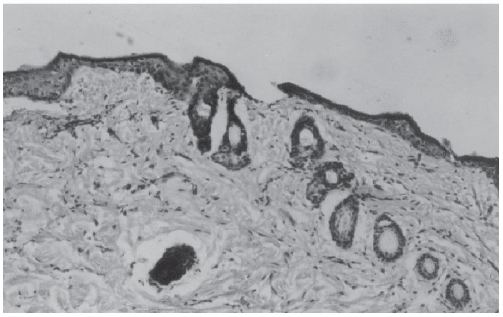

FIGURE 3. H&E photomicrographs of rabbit skin tissue after skin administration of clove oil (3% w/v), A, control group; B, clove oil treated group.

After gel composed of ibuprofen was used to the skin, the drug further diffuses to the circulatory system. The plasma concentration of ibuprofen at designated time after topical application was determined, as shown in Figure 2, a greater enhancement in skin by clove oil compared to the control group was found.

In vivo results demonstrated that clove oil showed a significant effect enhancing the absorption of ibuprofen in rabbits. Azone was reported to possess high transdermal enhancing ability for ibuprofen in vitro (Zhang & Liang, 1996). The skin permeation enhancement effects of 3% clove oil group and 2% azone group were similar. The AUC value of 3% oil group was 243% compared with the control group, indicating that the clove oil can enhance the absorption of ibuprofen in vivo. This result differed from the results of in vitro experiments, which showed a weaker enhancement of skin absorption by clove oil. The discrepancy may have been due to the different conditions in vitro and in vivo. The in vitro permeation study lacked elimination routes in terms of the vascular system and viable metabolizing enzymes (Fang, Leu, Hwang, & Cheng, 2004)

The GC-MS results (Table 3) showed major components from clove oils were terpenes with various carbon numbers. A variety of terpenes appeared to high transdermal enhancing ability, the permeation enhancement effect of clove oil maybe attributed to the terpenes. Among of the clove oil, the content of eugenol was nearly 82% and acetyleugenol was 8%, therefore, it is most probably that the effects of the oil on enhancing transdermal delivery of ibuprofenare attributed to eugenol and acetyleugenol.

There were no signs of skin irritancy after the application of the gel with clove oil at the test concentrations for 24, 48, and 72 hr. In addition, the studies performed in rabbits revealed no (1 week) skin irritancy potential for the ibuprofen-free gel with clove oil. The results indicated that clove oil might possess low skin irritation and therefore well tolerated by the rabbits.

In conclusion, the use of clove oil in ibuprofen transdermal formulations resulted in an improved transdermal penetration in vitro and in vivo. The clove oils may warrant further studies as suitable enhancers for the percutaneous absorption of ibuprofenand perhaps other pharmaceutical products.

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