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Cytotoxicity and penetration enhancement activity of essential oils from warming the interior medicinals with hot or warm property in terms of Traditional Chinese Medicine☆,☆☆

Yao Junhong[a], Jiang Qiudong[a], Cai Hao[b], Zhu Xuemin[a], Ma Min[a], Duan Jinao[a], Dong Jie[a,*], Chen Jun[a]

[a] Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Nanjing University of Chinese Medicine, Nanjing 210023, China
[b] School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China

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ABSTRACT

Objective: To investigate on the cytotoxicity and penetration enhancement effect of essential oils (EOs) from warming the interior medicinals (WIM) from Traditional Chinese Medicine (TCM).
Methods: EOs were extracted from WIM of Bichengqie (*Litseae Fructus*), Dingxiang (*Flos Syzygii Aromatici*), Hua- jiao (*Zanthoxyli Pericarpium*), and Xiaohuixiang (*Foeniculi Fructus*) with warm nature, and Ganjiang (*Rhizoma Zingiberis*), Gaoliangjiang (*Rhizoma Alpiniae Officinari*), Rougui (*Cortex Cinnamomi Cassiae*), and Wuzhuyu (*Fructus Evodiae Rutaecarpae*) with hot nature, respectively. Their chemical compositions were analyzed by gas chromatography–mass spectrometry (GC–MS). The cytotoxicity of the extracted eight EOs on HaCaT cells was measured and compared. Moreover, analyses of cell cycle and cell apoptosis were performed to investigate the cytotoxic mechanism. The transdermal penetration enhancement effects of the extracted eight EOs on ibuprofen were further compared by the modified Franz diffusion cell method.
Results: The most abundant constituents in the extracted eight EOs were determined to be monoterpenes, especially oxygen-containing monoterpenes. The HaCaT cell cytotoxicity of EOs from WIM with hot nature were significantly ($P=0.020$) higher than that with warm nature. Both ginger oil and zanthoxylum oil significantly induced G0/G1 phase arrestment in HaCaT cell cycle. For ginger oil from WIM with hot nature and zanthoxylum oil from WIM with warm nature, the main mechanisms of the cytotoxicity were found to be the induction of cellular necrosis and the cellular apoptosis, respectively. Furthermore, most of the tested EOs showed remarkable penetration enhancement activity on ibuprofen. However, no statistical significance ($P=0.18$) was found between penetration enhancement activity of EOs from WIM with warm nature and EOs from WIM with hot nature.
Conclusion: With the enhanced penetration activity, the extracted EOs from the WIM demonstrated their significant effect of the cytotoxicity on the skin cells.

1. Introduction

It is generally accepted that stratum corneum (SC), the outermost layer of the epidermis, is the principal barrier to drug permeation following transdermal drug delivery (TDD). In order to improve the percutaneous permeation rate of the drug, lots of strategies are utilized to alter the barrier function of SC [1]. One of the most widely used strategies is the use of penetration enhancers (PEs) which ideally cause a temporary and reversible reduction in the barrier function of SC [2].

Essential oils (EOs) which possess diverse and relevant biological activities have been widely used for several applications in pharmaceutical, cosmetic, agricultural, and food industries. EOs

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* Corresponding author.
E-mail addresses: 13915941917@139.com (D. Jie), chenjun75@163.com (C. Jun).

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and their volatile constituents have been used as PEs for decades of years [3]. They can facilitate the delivery of small drug compounds acrossing SC by interacting with the intercellular lipids through physical processes including extraction, fluidization, increased disorder, and phase separation. At present, natural PEs including EOs [3] and terpenes [4] have been increasingly used due to their better safety profile compared to chemically synthetic PEs. Moreover, it was also proved that the penetration enhancement capacities of the whole EOs were significantly higher than its main terpene components [5,6].

In Traditional Chinese Medicine (TCM), about 300 kinds of Chinese herbal medicines (CHMs) contain EOs. The association between penetration enhancement activities of EOs [7] and drug properties of CHMs containing EOs was significant, especially the four properties defined in terms of TCM theory. Furthermore, most EOs are from CHMs with warm or hot nature. Consequently, it is proposed that the screening of EOs as PEs can be performed based on drug characteristics of CHMs containing EOs.

It is found that EOs from warming the interior medicinal (WIM) from TCM are usually applied as PEs. The addition of 3% clove oil showed a significant permeation enhancement activity on ibuprofen and the enhancement ratio was determined to be 7.3 *in vitro* and 2.4 *in vivo* [9]. The skin permeation of trazodone hydrochloride was also found to be remarkably enhanced by EOs and the effect of fennel oil was significantly better than that of mentha oil, one of the most commonly used PEs [10]. However, to our knowledge, there is currently no information available about the comparison of different EOs from WIM.

Despite most PEs display fairly excellent performance for TDD, only a few of them have been approved for clinical application due to their skin toxicity or irritation issue. It is challenging to maintain an optimal balance between the safety and the potency of PEs in drug permeation. Therefore, in the present study, both cytotoxicity and penetration enhancement activity were evaluated and compared for eight different EOs from four WIM [Bichengqie (*Litseae Fructus*), Dingxiang (*Flos Syzygii Aromatici*), Huajiao (*Pericarpium Zanthoxyli Bungeani*), Xiaohuixiang (*Fructus Foeniculi*) with warm property and another four WIM [Ganjiang (*Rhizoma Zingiberis*), Gaoliangjiang (*Rhizoma Alpiniae Officinari*), Rougui (*Cortex Cinnamomi Cassiae*), Wuzhuyu (*Fructus Evodiae Rutaecarpae*)] with hot property. The influence of the WIM properties with pungent flavor on the cytotoxicity and penetrate enhancement activity of extracted EOs was further investigated and compared.

2. Materials and methods

2.1. Instruments

An Agilent 7890A gas chromatograph interfaced to an Agilent 5975C inert MSD with Triple-Axis Detector (Agilent Technologies, Palo Alto, CA, USA) was employed for the analyses of EOs. A Chromate-4300 microplate spectrophotometer (Awareness Technology Inc., Palm City, FL, USA) and a flow cytometer (Becton Dickinson, San Jose, CA, USA) were used for cytotoxicity studies. A Shimadzu HPLC system (Kyoto, Japan) consisting of a LC-20AT pump and a SPD-20A UV-VIS detector and a modified Franz diffusion cell device (Shanghai Kai Kai Technology, Shanghai, China) were employed for permeation studies.

2.2. Plant materials and reagents

Bichengqie (*Litseae Fructus*), Dingxiang (*Flos Syzygii Aromatici*), Huajiao (*Pericarpium Zanthoxyli Bungeani*), Xiaohuixiang (*Fructus Foeniculi*), Ganjiang (*Rhizoma Zingiberis*), Gaoliangjiang (*Rhizoma Alpiniae Officinari*), Rougui (*Cortex Cinnamomi Cassiae*), Wuzhuyu (*Fructus Evodiae Rutaecarpae*) were all purchased from the Nanjing Medicinal Material Company (Nanjing, China). All the crude herbs were authenticated by Prof. Yue Wei from School of Pharmacy, Nanjing University of Chinese Medicine. The voucher specimens were kept in the Herbarium of School of Pharmacy, Nanjing University of Chinese Medicine (Nanjing, China).

Ibuprofen, propylene glycol (PG), isopropyl alcohol (IPA), dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Azone was obtained from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). Acetonitrile with HPLC-grade obtained from Tedia Company Inc., (Fairfield, OH, USA) and deionized water was purified by an EPED super purification system (EPED, Nanjing, China). Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were purchased from Gibco BRL (GrandIsland, NY, USA). AnnexinV-FITC/PI Apoptosis Detection Kit, propidium iodide (PI), and DNA content Quantitation Assay were obtained from Nanjing KeyGen Biotech Co., Ltd., (Nanjing, China). All other reagents were used commercially available and of analytical grade.

2.3. Animals

Male Sprague–Dawley rats (180–220) g were obtained from Shanghai Jiesijie Laboratory Animal Co., Ltd., (Shanghai, China) with the license number of SCXK (Shanghai) 2013–0006. The animals were acclimatized for at least 1 week in a 12 h light/dark cycle with free access to standard chow and water. They were fasted for 12 h before experiments with the exception of water. Animal experiments were performed in accordance to the Principles of Laboratory Animal Care and Use in Research (Ministry of Health, Beijing, China). The protocols of animal experiments were approved by the Animals Ethics Committee of Nanjing University of Chinese Medicine.

2.4. Cell lines

HaCaT (human epidermal keratinocytes) cell lines were obtained from Nanjing KeyGen Biotech Co., Ltd., (Nanjing, China). The cells were incubated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin in a humidified incubator at 37 °C and 5% CO_2. Culture medium was replaced every other day. For all experiments, cells were seeded in a culture dish until adhere to 60–80% confluency. In addition, the control cells were treated with 1% (v/v) DMSO only.

2.5. Extraction of EOs

EOs were extracted by the steam distillation method.

Briefly speaking, the dried pericarp or rootstock of those CHM samples was ground into powders with a mixer. For each sample, the pulverized herb was accurately weighed and then transferred into a 1000 mL round-bottomed flask. A certain multiple (mL/g) of distilled water was added, and the sample was soaked for 1 h and subjected to 5–9 h of steam distillation using a Clevenger-type apparatus. Then the oil layer cooled to room temperature was transferred to a 5 mL measuring flask. A little anhydrous sodium sulfate was added to the sample to remove moisture. After centrifuge to remove sodium sulfate, the resultant EO was stored in an air tight bottle and kept refrigerated until use. EOs extracted from Bichengqie (*Litseae Fructus*), Dingxiang (*Flos Syzygii Aromatici*), Huajiao (*Pericarpium Zanthoxyli Bungeani*), Xiaohuixiang (*Fructus Foeniculi*) with warm property were named cubeb oil, clove oil, zanthoxylum oil, and fennel oil, respectively. EOs extracted from Ganjiang (*Rhizoma Zingiberis*), Rougui (*Cortex Cinnamomi Cassiae*),

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