ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. X:X (2023) XX-XX

records of natural products

Evaluation of *Ligusticum jeholense* Extracts for Skin Lightening

Lihui Zhou [1†], Wenyu Su [2†], Xiaoyuan Zhang [1], Chaofeng Yang [1] and Dan Lu [1*]

[1] *School of Pharmaceutical Sciences, Jilin University, Fujin Road 1266, Changchun, Jilin 130021, China*

[2] *Jilin Province Jilin Hospital of Integrated Traditional Chinese and Western Medicine, Jilin 132012, China*

(Received July 11, 2023; Revised September 21, 2023; Accepted September 23, 2023)

Abstract: Natural materials in facial prescriptions has been practiced for thousands of years in China. Natural plant extracts are increasingly used in skin-whitening products. We found Gao-Ben (the rhizome and root of *Ligusticum jeholense* Nakai et Kitag, GB) was a common herb used in external whitening prescriptions by retrieving ancient Chinese medicine texts and modern traditional Chinese medicine(TCM) documents. Then, the functions of GB in topical application prescriptions for skin lightening were investigated. The essential oil of GB (GBO) were extracted by supercritical CO_2 fluid extraction (SFE-CO_2), with the yield being 1.5%. The oil ingredients were analyzed by gas chromatography-mass spectrometry (GC-MS), and benzyl alcohol and ligustilide were major constituents, their relative contents were 55.3% and 35.7%. Then the enhanced penetration effects of GBO on test compounds, ferulic acid and ligustrazine were tested using the Franz diffusion cell. Finally, the changes in morphological features of rat skin treated with GBO were researched by computer-aided image analysis of haematoxylin and eosin (H&E) staining. This study indicates GBO can enhance skin penetration by changing the structure of the stratum corneum barrier. Therefore, the active components could be absorbed through the topical skin and exert the whitening effect.

Keywords: *Ligusticum jeholense*; extract oil; skin-hyperpigmentation; transdermal; promotor.

1. Introduction

Ligusticum jeholense Nakai et Kitag is a member of the Apiaceae plant, its rhizome and root has been used as the traditional medicine Gao-Ben (Korean name, Go-Bon) in China and Korea. Modern studies have indicated that Gao-Ben (GB) has anti-inflammatory, anti-osteoporosis and antioxidant activities and shows good curative effects on cardiovascular and cerebrovascular diseases, hypertension, various pain disorders, and Alzheimer's disease [1-4]. In the past decade, various compositions, triterpenoids, flavonoids, coumarins, fatty acids, steroids, and saccharides have been reported [2,5,6].

GB is a common herb in traditional Chinese medicine (TCM) prescription both oral and external. The history of GB external use can date back to the Jin Dynasty (317-420). It was initially only used as a deodorant to improve body scent [7] and was then widely used in topical prescriptions until the Tang

* Corresponding author:E-Mail: ludan@jlu.edu.cn

The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products Month-Month 202x EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.416.2306.2819
Available online: September 27, 2023

Dynasties (618-907)—the strongest and most flourishing era in the history of China. With social prosperity and scientific as well as technological progress, TCM achieved rapid development during this period [8]. Starting from Qian Jin Yao Fang (652), the first encyclopedia of TCM, facial medicine was categorized as a new class for the first time in the book. After that, most of the facial medicine contained GB [8,9], which proved GB playing a special role in skin health.

Today, skin-whitening products are gaining interest due to the fact that they are perceived to be milder and safer than fully synthetic products [10]. Over the past 2000 years and more, the beauty experience has grown substantially and increasingly matured in TCM. Taking a comprehensive review in TCM facial prescriptions aids both innovation and development for the skin-whitening industry. The development of information and computer technology creates the possibility of mining numerous sources and rich data of TCM. Skin whiten prescriptions of TCM can not only be used to lighten skin tone, but also treat over-pigmentation in clinics. The increasing interest and demand for skin whitening products globally, particularly in Asia, have necessitated rapid advances in research on skin whitening products used in TCM [11].

In this paper, whitening prescriptions had been retrieved from ancient Chinese medicine texts and modern documents. And the herb varieties and usage frequency in the prescriptions were analyzed by the Traditional Chinese Medicine Inheritance Support System (TCMISS). The result showed that GB was second only to Bai-Zhi (root of *Angelica dahurica*) and appeared in transdermal whiten formulae. Phytochemical studies on GB suggested it contains ferulic acid [5], which may lighten age spots because it inhibits cells' ability to produce melanin [12-14]. GB is an aromatic herb, the volatile oils are its main active components [15]. The essential oils of some plants have the effect of increasing skin permeability similar to modern transdermal drug delivery systems [16,17]. We conducted this study to verify if essential oil of GB (GBO) can enhance skin penetration. Here, the supercritical CO_2 fluid extraction (SFE-CO_2) method was used to extract the GBO. SFE-CO_2 technology has the advantages of a short extraction time, high yield of oils, simple processing, and low operating temperature, which will not affect the natural activity of heat-sensitive substances [18]. To study the effects of GBO on the transdermal amount of test compounds, transdermal tests were performed using the Franz diffusion cell in vitro, which is a widely used instrument to evaluate in vitro drug permeation [19, 20]. Ferulic acid and ligustrazine were used as two test compounds owing to the fact that they are functional constituents of whitening herbs. The morphologic changes of skin treated with GBO were assessed by computer-aided image analysis of haematoxylin and eosin (H&E) staining performed according to standard procedures [21]. Skin experiments suggested that GBO could enhance percutaneous absorption.

2. Materials and Methods

This study is conducted by following two steps of research procedures. First, we conducted corpus analysis of TCM prescription. The findings of the first step serves as the basis for the second research questions.

2.1. TCM Prescription Gathering and Analysis

Classic TCM prescriptions were collected from TCM texts from Tang Dynasties (600AD) to Qing Dynasties (1910 AD). Modern prescriptions were obtained from digital databases: CNKI (https://www.cnki.net/), Wanfang Data Knowledge Service Platform (http://www.wanfangdata.com.cn/), and VIP Database (http://qikan.cqvip.com/), plus the website of China National Intellectual Property Administration.

Keywords used in searching ancient texts are the following classical Chinese characters with meaning of ‘complexion dark’ or ‘skin black’: *gǎn*(黕), *gǎn*(皯), *àn*(黯), *lí*(黧), *hēi*(黑). Keywords used in searching modern Chinese medicine prescriptions are: ‘Fleck’, ‘aestates’, ‘chloasma’, ‘chromatosis’, ‘skin lightening’.

Analysis platform was *Traditional Chinese Medicine Inheritance Support System* (V2.5), provided by Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

2.2. GBO Extracting and Analysis

The dried rhizome and root of GB used in this study was purchased from Jishen Pharmacy Co., Ltd. (Jilin, China) and identified by Prof. Guangshu Wang (School of Pharmaceutical Sciences, Jilin University, China). The material was pulverized and passed through BS 20 mesh (<1 mm pore size) for later experimentation.

The extraction of GBO was carried out using SFE-CO_2 equipment (HA221-50-06, Nantong Huaan Experimental Instrument Ltd., China). After CO_2 was condensed in the refrigerator storage tank, it was driven into the kettles through a high-pressure pump when the temperature reached 60℃ in extraction kettle Ⅰ, 60℃ in separation kettle Ⅰ and 50℃ in separation kettle Ⅱ. The flow rate of CO_2 was set to 20 L/h/kg. The extraction cycle was started, and a constant temperature or pressure was maintained when the pressure of the extraction kettle and separation kettle was 35 MPa and 6 MPa, respectively. After 2 hrs, the extract was collected from separation kettle Ⅰ and then dehydrated with anhydrous sodium sulfate. For each experiment, a stainless steel extraction vessel (2 L) was loaded with 1 kg of sample. The GBO was kept in an airtight container at 4°C.

This study performed gas chromatography-mass spectrometry (GC-MS) analysis on a Shimadzu GC-2010 gas chromatography instrument with a Shimadzu 2010 mass spectrometer and DB-5 capillary column (30 m×0.32 mm×0.25 μm). The GC oven temperature was set as follows: from 60℃ to 280℃ at a rate of 12℃/min and then held for 10 mins. The carrier gas was at a flow rate of 1.0 mL/min by using high-purity nitrogen. The split ratio was 30:1. The mass spectrometer was run in Electron-Impact mode. The scan range was 20-550 amu. The scan rate was 0.5 s/scan. The temperatures at the interface and ionization source were 280℃ and 230℃, respectively. The chemical compositions of GBO were qualitatively analyzed by comparing mass spectra with the National Institute of Standards and Technology (NIST 2008) database and standard samples.

2.3. Transdermal Permeation Effect of GBO

2.3.1. Drugs and Chemicals

Ligustrazine and ferulic acid were obtained from Sichuan Weikeqi Technology Co., Ltd., China. Propylene glycol was purchased from Tianjin Bailunsi Chemical Reagent Co., Ltd., China. Azone and trypsin were supplied by Shanghai Macklin Biochemical Co., Ltd., China. Methanol of high-performance liquid chromatography (HPLC) grade was obtained from Thermo Fisher Scientific (China). Pentobarbital sodium was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd., China. All other chemicals used were of analytical grade.

2.3.2. Animals

A total of 16 healthy male Sprague-Dawley rats weighing 220±10 g were obtained from the Experimental Animal Center of Jilin University (Jilin, China), certificate NO. SCXK (Liao) 2015-0001. All rats were kept in cages containing adequate food and water at a constant temperature (22±2°C), humidity (50±10%), and suitable light (12 h light/dark cycle, lights on at 8:00 a.m.). All the experimental procedures were approved by the Committee on Animal Research of Jilin University and performed in full compliance with international practices for animal care and use.

2.3.3. Test Compounds Analyzing

The HPLC system for analyzing compound concentrations was equipped with an SPD-10A variable wavelength ultraviolet absorbance detector, two LC-10AT pumps, and a computer integrating system (N2000, Zhejiang, China). An Inertsil ODS-3 column (4.6 mm×250 mm i.d., 5 μm particle size; Japan) was used to determine the contents of ligustrazine and ferulic acid. The mobile phase was a mixture of methanol/0.1 mol/L acetic acid (35/65, v/v) at a flow rate of 1 mL/min. The detection wavelength of both was 280 nm. Quantified samples were filtered through a 0.22 μm membrane prior to manual

injection into the HPLC system. The calibration curves for ligustrazine and ferulic acid were linear over the range of 1-400 μg/mL.

2.3.4. Preparation of Skin and Stratum Corneum (SC)

The skin was prepared under extreme caution to reduce overall stratum corneum (SC) damage, and the abdominal skin of rats was shaved under general anesthesia with ether inhalation. Rats were sacrificed 24 hrs after shaving to reduce redness or blemishes.

The shaven skin was then removed from the dead rats. The fat and other tissues adhering to the skin underneath were carefully wiped. The full thickness of the skin was cleaned three times in succession with isotonic 0.01 M phosphate buffer solution (PBS, pH 7.2), wrapped in aluminum foil, and stored at -20°C (used within 2 weeks).

At room temperature, the dead rat skin was treated with 0.4% (w/v) trypsin solution for 10 hrs. The SC samples were divided by using a cotton swab moistened with double distilled water. The sheets were washed with water and dried in a vacuum desiccator.

2.3.5. Percutaneous Penetration Experiment

A vertical Franz diffusion cell (Gongyi Yuhua Instrument and Equipment Co., Ltd., China) with a receptor chamber volume of 20 mL (V) and a diffusional area of 1.77 cm^2 (A) was used to test the percutaneous penetration enhancement function of the GBO. Applied saturated suspension test compounds to ensure maximum thermodynamic activity and maintain sinking conditions. The control group fed with vehicle solution, a propylene glycol: water (78:22, v/v) vehicle containing 0.5 g/mL of test compounds. The experimental groups fed liquid included 1%, 3%, and 5% GBO in the vehicle. The positive groups fed liquid included 1%, 3%, and 5% azone in the vehicle.

The epidermis was caught between the diffusion cells with the SC side up and the dermal side next to the receiver compartment with isotonic PBS (pH 7.2). The receptor cells were kept at a constant temperature of 32±0.5°C, and the solution was stirred in the receptor chambers constantly at 400 r/min. Then, the skin was given 3 mL of feed liquid at varying concentrations. The control was treated with the vehicle. Samples (Vi=1 mL) were obtained from the receptor chambers at scheduled times (1, 2, 4, 6, 8, 10, 12 and 24 hr) post dosing and added to an equivalent volume of buffer solution. Then, the compounds concentrations (C_i, μg/mL) of each sample were assayed at once using the HPLC method described in section test compounds analyzing.

2.3.6. Skin Absorption Experiment

The SC samples were pulverized in a mortar with a pestle. One milliliter of varying concentrations of GBO in propylene glycol/water (78/22, v/v) containing 0.5 g/mL of test compounds was added to 10 mg of ground SC with frequent vortexing. The control group was only treated with the vehicle. The mixture was left for 12 hrs at 32°C. The supernatant was obtained by centrifugation at 10,000 r/min for 10 min, and then the compounds content in it was analyzed. The amount of compounds absorbed on the SC was calculated by subtracting the amount of the supernatant from the starting solution. The partition coefficient (K) equals the ratio of compound concentration in SC to the vehicle. Each measurement was performed in triplicate, and the standard deviation was calculated.

2.3.7. Data and Statistical Analysis

The parameters of the skin penetration study were obtained by plotting the relationship between the cumulative amount of skin penetrating drugs and time (h).

Lag time (Tlag, h) and steady-state flux (Jss, mg/cm^2/h) were obtained from the x-intercept and slope of the linear portion of the plot (between 6 h and 12 h), respectively. The cumulative amount of compound penetration per unit area of skin in 24 hours (Q, μg/cm^2) was calculated based on the concentration in the receptor compartment. To compare the enhancement of different penetration

enhancers, the enhancement ratio (ER) was also calculated. Q, ER and diffusion parameter (D/h^2) were calculated as follows:

$$Q = \frac{\left(C_n V + \sum_{i=1}^{n-1} C_i V_i\right)}{A}$$

$$ER = J_{ss}(with\ enhancer)/J_{ss}(without\ enhancer)$$

$$\frac{D}{h^2} = \frac{1}{6T_{lag}}$$

The data are expressed as the mean ± standard deviation, and the number of repetitions (n) is shown in relevant figures. The two-tailed Student's t-test was used to compare the two different conditions. On all accounts, $P<0.05$ was considered statistically significant.

2.3.8. Changes of Skin Histomorphology Induced by GBO

Rats were divided into eight groups: the blank, solvent, 1%, 3% and 5% azone groups, and 1%, 3% and 5% GBO groups. Each group contained two rats and was administered separately. The medicated solution was tested on the abdominal skin of rats. To prevent the rats from licking the medicine applied on their stomachs, animals were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) to fall asleep before the application of drugs [22]. The medicine was applied on the skin for 10 hrs. Rats were killed by anaesthetization with ether inhalation.

Tissue samples (2×2 cm) were obtained from the red circle of the abdominal skin, fixed in 4% paraformaldehyde for 48 h, and paraffin-embedded. By dewaxing, dehydration, staining, and microscopic examination, tissue sections were prepared. The structure of the SC was observed under a light microscope (magnification, ×100).

3. Results and Discussion

3.1. TCM Formulae Gathering and Analysis

TCMISS is a data mining tool that focuses on data mining and analysis of TCM prescriptions to explore potential therapeutic strategies. Here, 23 typical ancient Chinese medicine texts were selected owing to their authors are distinguished TCM doctors and representatives of different schools, and were masterpieces of different dynasties from Tang Dynasty (618 AD) to Qing Dynasty (1912 AD) of China. Modern prescriptions were obtained through searching digital databases. A total of 234 transdermal whiten prescriptions were collected, 206 from 19 ancient codes and 28 from present-day literatures. Obviously, TCM for external use achieved rapid development during Tang Dynasties, 85 skin-lightening formulae were recorded in two texts of this dynasty (Table 1). 344 herbs were involved in these topical formulae for treating skin hyperpigmentation. The top 3 most frequently used herbs were Baizhi, GB, Baifuzi, and the frequencies were 48.3%、41.5% and 35.5%, respectively. Apiaceae family plants are the largest and the most important group in external skin-lightening formulae, 5 of top 20 as well as 3 of top 5 come from this family.

Table 1. The sources and numbers of the formulae

Dynasty （Year）	Sources	Chinese names	English names	Numbers
Tang（618-907）	*Qiān Jīn Fāng*	千金方	*Essential Recipes for Emergent Use Worth A Thousand Gold*	56
	Wài Tái Mì Yào	外台秘要	*Medical Secretes of an Official*	29
BeiSong（960-1127）	*Yī Xīn Fāng*	医心方	*Ishinpo*	7
	Tài Píng Shèng Huì Fāng	太平圣惠方	*Taiping Holy Prescriptions for Universal Relief*	40
	Shèng Jì Zǒng Lù	圣济总录	*General Medical Collection of Royal Benevolenc*	11
NanSong (1127-1279)	*Yáng Shì Jiā Cáng Fāng*	杨氏家藏方	*Yang's Familly Formula*	2
Yuan（1279-1368）	*Shì Yī Dé Xiào Fāng*	世医得效方	*Effective Formulae Handed Down For Generations*	2
	Yù Yuàn Yào Fāng	御院药方	*Imperial Academy Formula*	4
	Wèi Shēng Bǎo Jiàn	卫生宝鉴	*Health Treasure Formula*	1
Ming（1368-1644）	*Wài Kē Zhèng Zōng*	外科正宗	*Orthodox Manual of External Diseases*	1
	Biàn Mín Tú Zǔan	便民图纂	*Convenience drawing compilation*	1
	Pǔ Jì Fāng	普济方	*Prescriptions for Universal Relief*	25
	Wèi Shēng Yì Jiǎn Fāng	卫生易简方	*Hygienic Easy to Simplify Formula*	1
	Xiāng Lián Rùn Sè	香奁润色	*Women beauty health monograph*	21
Qing（1644-1912）	*Wài Kē Zhèng Zhì Quán Shū*	外科证治全书	*Surgical Syndrome and Treatment Complete Book*	1
	Wén Táng Jí Yàn Fāng	文堂集验方	*empirical prescription*	1
	Yàn Fāng Xīn Biān	验方新编	*New Compilation of Empirical Formulas*	1
	Wài Zhì Shòu Shì Fāng	外治寿世方	*Prescription for external treatment to benefits the world*	1
	Qí Xiào Jiǎn Biàn Liáng Fāng	奇效简便良方	*A Wonderful and Convenient Prescription*	1

the People's Republic of China (1994 -)	*https://www.cnki.net/*	中国知网	*China National Knowledge Infrastructure*	10
	http://www.wanfangdata.com.cn/	万方数据知识服务平台	*Wanfang Data Knowledge Service Platform*	
	http://qikan.cqvip.com/	维普中文期刊全文数据库	*China Science and Technology Journal Database*	
	https://www.cnipa.gov.cn/	国家知识产权局	*State Intellectual Property Office*	18

Table 2. Compounds identified from the GBO

No	Compound[a]	Formula	MW	RRI[b]	RRI[c]	Content (%)
1	Sabinene	$C_{10}H_{16}$	136.23	964	941-975[a,b,c,d]	0.060
2	Crithmene	$C_{10}H_{16}$	136.23	1047	1031-1074[a,b]	0.054
3	α-terpinoene	$C_{10}H_{16}$	136.23	1078	1045-1097[a,b]	0.006
4	β-Elemene	$C_{15}H_{24}$	204.35	1387	1382-1391[a,b,c]	0.071
5	Aromadendrene	$C_{15}H_{24}$	204.35	1439	1436-1463[a,b,d]	0.024
6	β-Farnesene	$C_{15}H_{24}$	204.35	1448	1422-1458[a,b]	0.118
7	α-Bisabolene	$C_{15}H_{24}$	204.35	1452	1495-1509[a]	0.049
8	Humulene	$C_{15}H_{24}$	204.35	1466	1432-1487[a,b]	0.071
9	α-Curcumene	$C_{15}H_{22}$	202.34	1472	1453-1473[a,b]	0.065
10	β-Selinene	$C_{15}H_{24}$	204.35	1483	1452-1493[a,c,d]	0.311
11	α-Selinene	$C_{15}H_{24}$	204.35	1489	1485-1517[a]	0.193
12	Cuparene	$C_{15}H_{22}$	202.34	1496	1488-1500[a,b]	0.036
13	Sesquiphellandrene	$C_{15}H_{24}$	204.35	1501	1514-1561[a]	0.179
14	2,6-Octadiene, 2,6-dimethyl-	$C_{10}H_{18}$	138.25	1509	-	0.020
15	Spathulenol	$C_{15}H_{24}O$	220.35	1521	1545-1575[a]	0.744
16	Myristicin	$C_{10}H_{10}O_4$	194.18	1529	1509-1532[a,b]	0.057
17	cis,trans-4-methyl-3-oxabicyclo[4.4.0]decane	$C_{10}H_{18}O$	154.25	1535	-	0.176
18	1-Propanone, 1-(2,4-dimethylphenyl)-	$C_{11}H_{14}O$	162.23	1544	-	0.459
19	1(3H)-Isobenzofuranone, 3-butylidene-	$C_{12}H_{12}O_2$	188.22	1558	-	1.324
20	5-Tetradecen-3-yne, (E)-	$C_{14}H_{24}$	192.34	1576	-	0.138
21	6-Butyl-1,4-cycloheptadiene	$C_{11}H_{18}$	150.26	1645	-	2.000
22	Benzyl Alcohol	C_7H_8O	108.14	1653	-	55.285
23	ligustilide	$C_{12}H_{14}O_2$	190.24	1685	-	35.679
24	Phenol, 2-octyl-	$C_{14}H_{22}O$	206.32	1708	-	0.044
25	3,5-Dodecadiyne, 2-methyl-	$C_{13}H_2O$	176.30	1933	-	0.266
26	Palmitic acid	$C_{16}H_{32}O_2$	256.42	1940	1958-1971[a,b,e]	0.554
27	Falcarinol	$C_{17}H_{24}O$	244.37	1980	1997[b]	0.067
28	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	2092	2075-2094[a,b]	0.030
29	Linoleic acid	$C_{18}H_{32}O_2$	280.45	2095	2095-2131[a,b,e]	1.080
30	Oleic Acid	$C_{18}H_{34}O_2$	282.46	2113	2090-2175[b,e]	0.619
31	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280.45	2199	2199[b]	0.149
32	10,12-Octadecadiynoic acid	$C_{18}H_{28}O_2$	276.41	2202	2202[b]	0.070

[a]Compounds are listed in order of their elution from a DB-5 column; [b]RRI: retention indices calculated against C_8-C_{30} n-alkanes mixture on DB-5 column; [c]RRI: retention indices of literature reports: a) NIST Standard Reference Database Number 69; b) Pubchem database; c) [28], d) [29], e) [30].

3.2. GBO Extracting and Analysis

Optimization of the extraction conditions (pressure, temperature, and time) of SFE-CO_2 was carried out for maximum oil yield from GB powder. The GBO yield reached 1.5% (v/w, mL/g) with the adopted method. The total ion chromatogram of the obtained oil is shown in Figure S1. The volatile compounds were recognized by comparing the existing mass spectra with standard spectra from the NIST 2008 mass spectral library and by the Kovats retention indices calculated for each peak with reference to the normal alkane C_8-C_{30} series. The method for the relative contents in percentage of the constituents in the sample analyzed by gas chromatography was area normalization. The major constituent of the GBO was benzyl alcohol (55.29%), which is a common additive to cosmetics for preservation and reducing viscosity. Followed by ligustilide (35.68%), a natural benzoquinone derivative, has a wide range of pharmacological properties[23-25]. Extracting the GBO with SFE-CO_2 method achieved higher oil yield (1.5mL/100g) than by hydrodistillation, 0.17mL/100g and 0.11 g/100g [26,27]. Compared with the oil extracted with petroleum ether by Soxhlet extractor [21]. Furthermore, the constituents of GBO obtained by the SFE-CO_2 method have more variety, totally 32 kinds of ingredients were recognized. The names, RRI values and contents of the 32 identified compounds are listed in Table 2.

3.3. Skin Penetration Experiment

The enhancing effect of GBO on the transdermal absorption of rat skin was tested using Franz diffusion cells for 24 hrs. Taking the time as the X-axis and the cumulant of transdermal drug (Q) as the Y-axis, a cumulative penetration curve was drawn (Figure 1). The results showed that the cumulant of positive compound and two test compounds, ferulic acid and ligustrazine, were increased along with prolonged time during the experiment. The Q values of azone groups and GBO groups were significantly greater than those of the control groups. The transdermal amount of ferulic acid was positively correlated with GBO doses in the test. The Q values of ligustrazine of 1% GBO group and 3% GBO group were similar, the Q values of ferulic acid in 24 hrs of 5% GBO was higher than 3% azone groups. The permeation parameters of ligustrazine and ferulic acid are listed in Table 3. When comparing the enhancement ratio (ER) of each group, we found that the value 5% GBO on ferulic acid was very close to those of 1% and 3% azone groups. The results suggested that GBO did have the effect of promoting penetration of rat skin, it can enhance skin penetration by changing the structure of the stratum corneum barrier, although less than azone. Therefore, the active components, like ferulic acid, could be absorbed through the topical skin and exert the whitening effect. It is beneficial to the transdermal absorption of other whitening ingredients in the prescription to exert the skin-lightening effect.

The effects of GBO on the partition coefficient (K) of chemical substances in SC and vehicle systems were researched. The results showed that the K values of both test compounds increased in the azone and GBO groups (P<0.05), and there were no statistically significant differences among the azone and GBO groups. This suggests that GBO can promote compound partitioning in the SC, whose permeability barrier is mostly formed by saturated lipids. The fat-soluble compounds of GBO can affect the physical and chemical properties of the skin cuticle and easily penetrate the compounds.

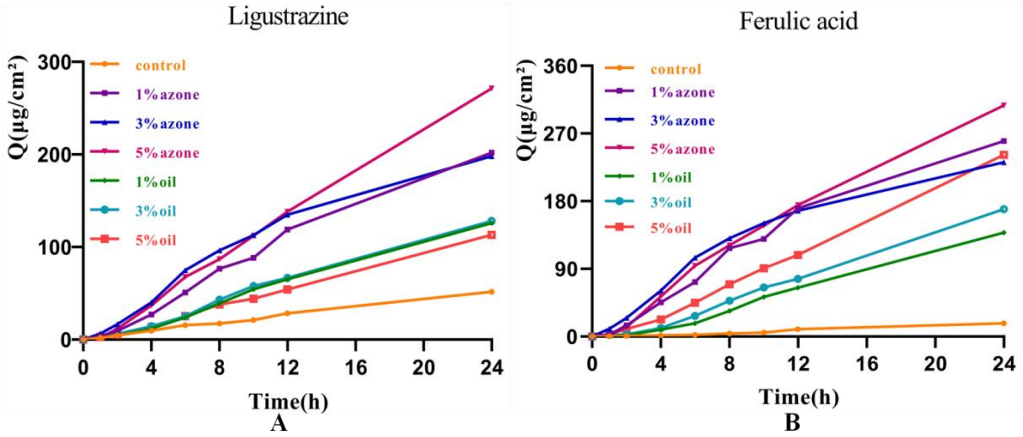

Figure 1. The cumulative penetration amounts of test compounds

Table 3. Permeation parameters of ligustrazine and ferulic acid.

Enhancer	J_{ss}(μg/cm²/h)		T_{lag}(h)		Q_{24}(μg/cm²)		ER	
	Ligustrazine	**Ferulic acid**	**Ligustrazine**	**Ferulic acid**	**Ligustrazine**	**Ferulic acid**	**Ligustrazine**	**Ferulic acid**
control	0.76±0.04	2.39±0.47	8.71±0.38	12.36±0.12	49.52±7.63	16.64±2.63	1.00	1.00
1% Azone	21.53±2.28*	30.64±4.19*	1.13±0.72*	0.93±0.33	207.48±34.68*	265.03±38.41*	4.61	6.77
3% Azone	19.63±2.03*	20.78±3.53*	1.21±0.32*	0.96±0.72*	184.25±29.13*	233.79±29.07*	3.40	6.26
5% Azone	23.66±2.33*	26.88±3.80*	0.75±0.22*	0.87±0.19*	276.87±40.21*	316.27±49.68*	5.88	7.02
1% oil	13.94±1.37*	16.13±2.75*	1.89±0.60	3.68±0.60*	125.82±19.27*	137.28±18.32*	2.73	2.97
3% oil	13.92±1.40*	16.5±2.91*	1.77±0.51*	3.17±0.24*	128.16±20.15*	171.34±27.15*	2.94	2.83
5% oil	9.26±0.84*	21.21±3.59*	1.83±0.34*	2.45±0.35*	114.29±16.53*	246.35±38.67*	1.02	6.40

Each value represents the mean±standard deviation (n=6), except for ER. *P<0.05, statistically significant difference between enhancers and control.

3.4. Changes of Skin Histomorphology

H&E staining showed that the skin structure of the control group was complete, and the SC as well as layers of epidermis were arranged tightly, neatly, and clearly layered. Keratin cells were tightly connected, and there were few keratin fragments (Figure 2A, B). The SC became loose and thin, keratin fragments increased, the intercellular space in the spinous layer became large, and the basal layer cells were loosely arranged after treatment with azone and GBO (Figure 2C to H). When the concentrations of azone and GBO reached 5%, the SC was loosened and peeled off (Figure 2E, H). These results suggest that the permeability enhancement of azone and GBO was related to the change in skin microstructure. As a transdermal drug delivery carrier, GBO can dilute the dense membrane structure formed by lipids, proteins and nonfibrin, weakening its barrier function. This will provide evidence for the further study of GBO as a transdermal drug delivery carrier.

The results of the in vivo promotion of rat abdominal skin showed that GBO could affect the structure of the SC, reduce the compactness and increase the degree of porosity of the SC. The results of H&E staining showed that the 5% GBO group and 5% azone group had similar effects on the structure of the SC, one of the penetration promotion mechanism of GBO may be similar to that of the positive compound azone, and the specific mechanism of the action and biochemical reaction of the permeation-enhancing effect needs to be further tested in the future.

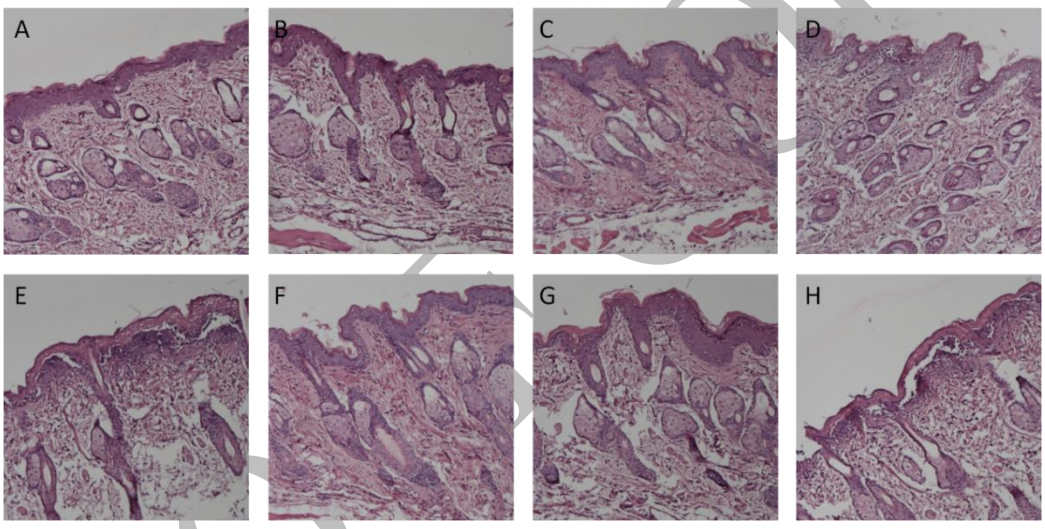

Figure 2. The H&E stainining of rat skin. (A) Normal. (B) Solvent blank. (C) 1% azone. (D) 3% azone. (E) 5% azone. (F) 1% GBO. (G) 3% GBO. (H) 5% GBO.

4. Conclusions

Classic prescriptions that TCM gave to the world were the result of refining hundreds years of clinical practice. Understanding the mechanisms of function is critical for selecting precision medicine, designing formulation, and improving the efficacy of therapy. This study conducted an in-depth analysis of the herb varieties and usage frequency in external skin-lightening formulae through TCMISS, found GB was the second most frequently used herb. A approach GC-MS was used to identify the chemical component of essential oil from *Ligusticum jeholense.* The oil was shown to contain sesquiterpene hydrocarbons, monoterpene hydrocarbons and fatty acids. After its extraction, skin whitening properties of the oil were investigated. GBs have dual functions of permeation enhancement and skin whitening activity, that may be the reason GBs are widely used in beauty prescriptions of TCMs. Our preliminary conclusion is that GB promotes the absorption of active ingredients and the integrative whiten effect of the formulae is greatly improved. Further studies are required to explore the efficacies and mechanisms of GB and TCM whiten prescriptions.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

ORCID
Lihui Zhou: 0000-0002-7377-5988
Wenyu Su: 0000-0002-1007-7955
Xiaoyuan Zhang: 0000-0002-0364-8692
Chaofeng Yang: 0009-0000-0734-5253
Dan Lu: 0000-0002-0311-5335

References

[1] S. M. He, Y. K. Chen, A. Zeng, C. M. Li and Q. Lu (2021). Research progress of ligustilide pharmacological action and mechanism, *J. Guangdong Pharmaceut. Univ*. **37**, 152-156.

[2] D. D. Huang, X. H. Ma, Y. M. Yang, Y. Y. Lu, L. Lin, T. T. Zhu, P. L. Lv and L. Jin（2017）. Market research and comparative study on genuine and conventionally used ligustici rhizoma et radix. *Chinese J. Inform. TCM*. **24**, 1-4.

[3] B. Kim, K. Lee, K. S. Chinannai, I. Ham, Y. Bu, H. Kim and H. Y. Choi（2015）. Endothelium-independent vasorelaxant effect of *Ligusticum jeholense* root and rhizoma on rat thoracic aorta. *Molecules* **20**, 10721-10733.

[4] X. Q. Lei, G. Li, L. Cheng, X. L. Wang and F. Y. Meng (2018). Identification of ligustici rhizoma et radix and its adulterants based on their chemical constituents by UHPLC-Q/TOF-MS combined with data mining, *J Pharm Biomed Anal*. **154**, 123-137.

[5] B. Zhang (2009). The study of chemical components in *Ligusticum jeholense* Nakai et Kitag, Changchun University of Chinese Medicine.

[6] D. M. Su, S. S. Yu and H. L. Qin (2005). New dmieric phthalide derivative from *Angelica sinensis*, *Acta Pharmaceut. Sinica* **40**, 141-44.

[7] M. R. Fu (2016). Study on the law of use of traditional Chinese medicine beauty prescription of Gehong in Eastern Jin Dynasty, *Sci. Technol*. **26**, 256.

[8] M. L, F. Z. Liu, Y. Du, Y. Y. Zhang, Y. Yang, J. Wang and Y. B. Li (2022). Analysis of compatibility rules of chinese medicine cosmetic external prescriptions by data mining technology, *New Chin. Medicin***. 54**, 16-22.

[9] L. J. Qi, J. Y. Dai and X. F. Liu (2022). Characteristics of facial medicines in important formulas worth a thousand gold pieces for emergency, *Henan Tradit. Chin. Medicin*. **42**, 526-529.

[10] M. Kanlayavattanakul and N. Lourith (2018). Skin hyperpigmentation treatment using herbs: A review of clinical evidences, *J. Cosmet. Laser. Ther*. **20**, 123-31.

[11] C. Y. Ko, J. Chao, P. Y. Chen, S. Y. Su, T. Maeda, C. Y. Lin, H. C. Chiang and S. S. Huang (2021). Ethnobotanical survey on skin whitening prescriptions of traditional chinese medicine in Taiwan, *Front. Pharmacol*. **12**, 736370.

[12] M. Strzępek-Gomółka, K. Gaweł-Bęben, A. Angelis, B. Antosiewicz, Z. Sakipova, K. Kozhanova, K. Głowniak and W. Kukula-Koch (2021). Identification of mushroom and murine tyrosinase inhibitors from *Achillea biebersteinii* Afan. Extract, *Molecules* **26**, 964-983.

[13] H. J. Park, J. H. Cho, S. H. Hong, D. H. Kim, H. Y. Jung, I. K. Kang and Y. J. Cho (2018). Whitening and anti-wrinkle activities of ferulic acid isolated from *Tetragonia tetragonioides* in B16F10 melanoma and CCD-986sk fibroblast cells, *J. Nat. Med.* **72**, 127-135.

[14] H. Maruyama, F. Kawakami, T. T. Lwin, M. Imai and F. Shamsa (2018). Biochemical characterization of ferulic acid and caffeic acid which effectively inhibit melanin synthesis via different mechanisms in b16 melanoma cells, *Biol Pharm Bull*. **41**, 806-810.

[15] S. M. Li, Y. Huang, F. Zhang, H. Ao and L. Chen (2022). Comparison of volatile oil between the *ligusticum sinese* Oliv. and *Ligusticum jeholense* Nakai et Kitag. based on GC-MS and chemical pattern recognition analysis, *Molecules* **27**, 5325.

[16] N. Laothaweerungsawat, W. Neimkhum, S. Anuchapreeda, J. Sirithunyalug and W. Chaiyana (2020). Transdermal delivery enhancement of carvacrol from *Origanum vulgare* L. essential oil by microemulsion, *Int. J. Pharm*. **579**, 119052.

[17] S. F. Ruan, Z. X. Wang, S. J. Xiang, H. J. Chen, Q. Shen, L. Liu, W. F. Wu, S. W. Cao, Z. W. Wang, Z. J. Yang, L. D. Weng, H. X. Zhu and Q. Liu (2019). Mechanisms of white mustard seed (*Sinapis alba* L.) volatile oils as transdermal penetration enhancers, *Fitoterapia* **138**, 104195.

[18] A. Piras, S. Porcedda, D. Falconieri, A. Fais, B. Era, G. Carta and A. Rosa (2022). Supercritical extraction of volatile and fixed oils from *Petroselinum crispum* L. seeds: chemical composition and biological activity, *Nat Prod Res*. **36**, 1883-1888.

[19] N. A. Charoo, A. A. Shamsher, K. Kohli, K. Pillai and Z. Rahman (2008). Improvement in bioavailability of transdermally applied flurbiprofen using tulsi (*Ocimum sanctum*) and turpentine oil*, Colloid. Surf. B Biointerface*. **65**, 300-307.

[20] G. Le Guyader, B. Do, V. Vieillard, K. Andrieux and M. Paul (2020). Comparison of the in vitro and ex vivo permeation of existing topical formulations used in the treatment of facial angiofibroma and characterization of the variations observed, *Pharmaceutics* **12**, 1060. doi: 10.3390/pharmaceutics12111060.

[21] B. Qin, C. N. Sun, L. Chen, S. Y. Wang, J. N. Yang, Z. Xie and Z. Shen (2021). The nerve injuries attenuate the persistence of psoriatic lesions. *J. Dermatol. Sci*. **102**, 85-93.

[22] Y. J. Sun, N. Zhang, Y. X. Qu, Y. J. Cao, J. H. Li, Y. W. Yang, T. Yang and Y. K. Sun (2020). Shuangxia decoction alleviates p-chlorophenylalanine induced insomnia through the modification of serotonergic and immune system, *Metab. Brain Dis*. **35**, 315-325.

[23] Q. Xie, L. Zhang, L. Xie, Y. Zheng, K. Liu, H. Tang, Y. Liao and X. Li (2020). Z-ligustilide: A review of its pharmacokinetics and pharmacology, *Phytother. Res*. **34**, 1966-1991.

[24] Z. Mao, L. Tian, J. Liu, Q. Wu, N. Wang, G. Wang, Y. Wang and S. Seto (2022). Ligustilide ameliorates hippocampal neuronal injury after cerebral ischemia reperfusion through activating PINK1/Parkin-dependent mitophagy, *Phytomedicine*. **101**, 154111.

[25] Q. Wu, J. Liu, Z. Mao, L. Tian, N. Wang, G. Wang, Y. Wang and S. Seto (2022). Ligustilide attenuates ischemic stroke injury by promoting Drp1-mediated mitochondrial fission via activation of AMPK, *Phytomedicine* **95**, 153884.

[26] Y. H. Li, H. Y. Li, Y. N. Wang and Y. Qiao (2022). Analysis of components of volatile oil extracted.from.Bupleuri by four methods, *Heilongjiang Animal Husband. Vet. Medicin.*. **02**, 117-122.

[27] Y. Y. Wang, Q. Hu, C. L. Yang, Y. Wang and Z. Su (2022). The components of volatile oil in leaves and fruits of Bamboo Nantian were analyzed by GC-MS combined with retention index method, *Chin. Food Additives* **33**, 197-207.

[28] J. Benites, D. Ríos, A. Guerrero-Castilla, C. Enríquez, E. Zavala, R.O. Ybañez-Julca, I. Quispe-Díaz, R. Jara-Aguilar, P. B. Calderon (2021). Chemical composition and assessment of antimicrobial, antioxidant and antiproliferative activities of essential oil from *Clinopodium sericeum*, a peruvian medicinal plant, *Rec. Nat. Prod.* **15**, 175-186.

[29] M.Y. Tian, Y. Hong, X. H. Wu, M. Zhang, B. Lin and Y. Zho (2020). Chemical constituents and cytotoxic activities of essential oils from the flowers, leaves and stems of *Zingiber striolatum* Diels. *Rec. Nat. Prod*. **14**,144-149.

[30] X. C, Zhang, L. Zhu, X. Y. Li, L. C. Liu and P. X. Lai (2021). Chemical composition, and evaluation of antibacterial, antibiofilm and synergistic effects with conventional antibiotics of essential oil from *Mallotus repandus, Rec. Nat. Prod.* **15**, 324-329.