Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
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ORIGINAL ARTICLE

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Study on the antipruritic mechanism of Zanthoxylum bungeanum and Zanthoxylum schinifolium volatile oil on chronic eczema based on H1R and PAR-2 mediated GRPR pathway

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Received 2 August 2021; Accepted 22 November 2022
Available online 1 July 2022

KEYWORDS
chronic eczema;
pruritus;
Zanthoxylum bungeanum;
Zanthoxylum schinifolium;
volatile oil

Abstract
Objective: To observe the antipruritic effect and mechanism of the volatile oil of Zanthoxylum bungeanum and Zanthoxylum schinifolium on chronic eczema to provide data support for clinical application and new drug development of Zanthoxylum bungeanum and Zanthoxylum schinifolium.
Methods: The model of chronic eczema was established by using 2-dinitrochlorobenzene (DNCB), and the composition and content of volatile oil in Zanthoxylum schinifolium and Zanthoxylum bungeanum was determined by gas chromatography-mass spectrometry (GC-MS). The antipruritic effect by (EASI) score of eczema area and severity index and scratching times was then evaluated. Then, the contents of histamine, gastrin-releasing peptide (GRP), interleukin-4 (IL-4), and immunoglobulin E (IgE) in serum of rats was determined by enzyme-linked immunosorbent assay (ELISA). The tissue morphology was observed by HE staining. The expressions of H1R, PAR-2, TRPV1, TRPA1, and GRPR was then detected by immunohistochemistry, Western blot, and QRT-PCR.
Results: The results revealed that there were differences in the composition of volatile oil between Zanthoxylum bungeanum and Zanthoxylum schinifolium. Compared to the model group, the medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium group significantly increased the difference of EASI score and scratching times, significantly decreased the concentrations of IL-4, IgE, GRP, and histamine, and significantly decreased the expression levels of H1R, PAR-2, TRPV1, and GRPR. The degree of inhibition on the pathological manifestations of chronic eczema was evident. There was no significant difference in antipruritic effect between the two groups. The expression of TRPA1 was inconsistent at the protein and gene level, which needs to be further researched.
Conclusion: The volatile oil of Zanthoxylum bungeanum and Zanthoxylum schinifolium can reduce the expression of H1R, PAR-2, TRPV1, and GRPR. The mechanism may be through the H1R and PAR-2-mediated GRPR pathway intervention to achieve the effect, both of which have the same antipruritic effect.

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https://doi.org/10.15586/aei.v50i4.486

Introduction

Chronic eczema is a common skin disease characterized by skin thickening, pigmentation, rough surface, and obvious pruritus. It has the characteristics of complex etiology, high incidence, long course of the disease, easily reoccurring, challenging to cure, etc., which acutely affects the quality of life of patients.[1] As in other parts of the world, the prevalence of eczema in Asian countries has been on the rise in recent years.[2] Atopic dermatitis (AD) has a significant and increasing prevalence in both pediatric and adult populations worldwide. In particular, AD affects people living in urban areas, raising concerns that the prevalence of AD will increase as Asian metropolitan areas grow in number and size.[3]

Eczema has a significant impact on the quality of life. The quality of life of patients with eczema decreases significantly as it affects the mental, physical, and overall health of the patients. People with eczema are more likely to suffer from depression, anxiety, and sleep disorders.[4] It also puts a huge financial burden on patients.[2]

Pruritus, one of the most acute symptoms of inflammatory dermatosis, is defined as "the urge to scratch" and is the leading cause of recurrent eczema.[5] In severe cases, the patient scratches the affected area of the skin until it causes bleeding.

There are mainly two neural pathways in pruritus signal transduction: non-histaminergic and histaminergic pathways.[6] The non-histaminergic path plays a significant role in chronic pruritus.[6] Endogenous/exogenous itching agents bind to their receptors and activate the downstream transient receptor potential vanilloid 1 (TRPV1) or transient receptor potential ankyrin 1 (TRPA1) through the phospholipase system.[6] Among the receptors, protease-activated receptors 2 (PAR-2) and protease-activated receptors 4 (PAR-4) are the most prominent ones.[7] The itching signal is then transmitted to the spinal cord, releasing the excitatory neurotransmitter gastrin-releasing peptide (GRP) to bind to gastrin-releasing peptide receptor (GRPR), and to conduct the signal to the brain.[6]

Modern medicine believes that chronic eczema is a delayed hypersensitivity induced by complex internal and external factors. The internal causes include genetic factors, immune factors, blood circulation disorders, endocrine changes, neurological factors, and so on.[8] External causes include environment, infection, diet, drugs, and so on.[8] Modern medicine often uses moisturizing creams, corticosteroids, immunosuppressants, antibiotics, antihistamines, laser therapy, and so on to treat eczema.[5] When compared to western medicine, traditional Chinese medicine has the advantages of fewer side effects and more accessibility to cure the root causes, so it has a particular developmental prospect.[9] Calamine lotion is one of the most widely used Chinese patent medicines for treating chronic eczema. The antipruritic effect is remarkable.[10] After the application of calamine lotion, the skin becomes dry, becomes difficult to care for, and the antipruritic effect is short.

Compared with calamine lotion, Zanthoxylum bungeanum oil for external use has certain advantages. The oil can nourish the skin, improve the skin barrier function and relieve itching.[11] Oil is better absorbed by the skin than water.[12] Zanthoxylum bungeanum volatile oil can also relieve pain, infection and other secondary damage caused by severe scratching of the skin. Zanthoxylum bungeanum oil has analgesic, anti-inflammatory, bactericidal, and other effects.[13]

The 2020 edition of Chinese Pharmacopoeia[14] contains research on Zanthoxylum bungeanum for relieving pain, and stopping the itching. It can treat eczema externally. Chen Ping[15] summed up the first 20 herbs for external use in the treatment of chronic eczema with high frequency and good efficacy, and Zanthoxylum bungeanum is one of them. The homology of medicine and food determines the safety of Zanthoxylum bungeanum to the human body. It is known that Zanthoxylum bungeanum oil is effective in relieving itching in the treatment of chronic eczema,[16] but its antipruritic mechanism is unclear.

The 2020 edition of Chinese Pharmacopoeia considers Zanthoxylum bungeanum and Zanthoxylum schinifolium as the source of Zanthoxyli pericarpium. Yang[17] shows significant differences in the content, composition of volatile oil between Zanthoxylum bungeanum and Zanthoxylum schinifolium. Whether the two antipruritic effects are the same, remains to be further studied.

Therefore, the purpose of this study is to explore the possible mechanism of Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil in relieving itching by observing the scratching and the expression of PAR-2, TRPV1, TRPA1 and GRPR.

Materials and methods

Materials

Experimental animal

SPF grade Wistar adult male rats, weighing 140g-160g, were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., and the observed animal license number is SCXK (Beijing) 2016-0006. It was raised in the Experimental Animal Center of Shandong University of Traditional Chinese Medicine. Before the experiment, the rats were fed adaptively for one week, and was then left to eat and drink freely. We have kept the temperature in the laboratory at 22-25°C.

Drugs and reagents

Zanthoxylum schinifolium (batch number: 1811200682) and Zanthoxylum bungeanum (batch number: 2003240012) was purchased from Miaozhou Huzhu Pharmaceutical Co., Ltd. 2,4-Dinitrochlorobenzene (DNCB) (batch number: 201906281) was bought from Chengdu Aikeda Chemical Reagent Co., Ltd., and acetone (batch number: 20140426) was purchased from Tianjin Fuyu Fine Chemical Co., Ltd. Compound calamine lotion (batch number: 200350) was purchased from Shanghai Yunjia Huangpu Pharmaceutical Co., Ltd., and Rb an HRH1 (batch number: AB09051540) from Bioss. Rabbit TRPV1 antibody (batch number: PAB45899) was purchased from Bioswamp, PAR-2 Rabbit mAb (batch number: 4000001246), TRPA1 Rabbit pAb (batch number: 1152380101), and GRPR Rabbit pAb (batch number: 0089320201) were purchased from ABclonal.

Experimental method

Preparation of rat model of chronic eczema

The 2,4-Dinitrochlorobenzene smear on the back to make a model of chronic eczema is a classical method.[18,19] After adaptive feeding, 80 Wistar male rats were divided into blank (n = 8) and model groups (n = 72). Following the method of Jegal J,[20] on the first day of the experiment, all rats were shaved on the abdomen in an area of 4.0 cm^2. On the second day, the abdominal skin of the model group was smeared with 100 μL of 7% DNCB acetone solution, and the abdominal skin of the blank group was smeared with 100 μL of normal saline. The two groups were repeated once on the third day. On the 6th day, the back of all rats were shaved in an area of 9 cm^2. On the seventh day, the back skin of the model group was smeared with 200 μL of 1%DNCB acetone solution. The back skin of the blank group was smeared with 200 μL normal saline, once every three days - a total of 6 times. Around 48 hours after the last modeling, the skin lesions were observed to have scales, blood scabs, and brown erythema, which suggested that the model was successful.

The remaining 72 rats in the model group were divided into model group (n = 8), model solvent group (n = 8), compound calamine lotion group (n = 8), high dose group (n = 8), middle dose group (n = 8), low dose group (n = 8) of Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil.

Extraction and composition Analysis of volatile Oil

According to the method of Lin Y[21,22] crushed Zanthoxylum bungeanum was sifted through 20 mesh, weighed 600 g precisely, was placed in 10000 mL flask, 12 times the amount of water was added along with several glass beads, shaken and mixed, and soaked for 20 minutes. Connected with volatile oil collector and reflux condensation tube, the volatile oil was extracted by heating and micro-boiling Zanthoxylum bungeanum for 6 hours. The collected volatile oil was extracted with 50 ml ether, dried ether, dried anhydrous sodium sulfate overnight, and the yield of volatile oil was calculated.

$$\text{Yield of volatile oil} = \frac{\text{volatile oil quality (g)}}{\text{Zanthoxylum bungeanum powder quality (g)}} \times 100\%.$$

The volatile oil components of Zanthoxylum bungeanum and Zanthoxylum schinifolium was calculated by gas chromatography-mass spectrometry (GC-MS). The precise weighing of Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil each 50 μL to get two sample solutions.

GC-MS detection conditions[23]: An Agilent HP-5 capillary column (0.25mm × 15m, 0.25mm) was used. The injection volume was taken as 1 mL, with a flow rate of 1 $mL{\cdot}min^{-1}$. The split ratio was considered as 10:1. The forward sample temperature was set as 250°C. The auxiliary heating temperature was 280°C. The programmed temperature was set as follows: an initial temperature of 50°C, held for 2 min, and raised to 230°C at the rate of 10°C / min, contained for 3 min. The total analysis time was 23 min, the temperature of the ion source 230°C, and the temperature of the four-stage rod 150°C. The scanning range was m/z 35-590, and the solvent delay time was set as 2 min.

A computer was used to search the spectral library NIST17.L and delete the components with a matching degree of less than 80%. Combined with the retention time, the chemical composition in the essential oil was determined. The relative quantification of chemical components by the chromatographic peak area normalization method was also carried out.

External administration

The prepared the volatile oil of Zanthoxylum bungeanum and Zanthoxylum schinifolium was divided into 4% (high dose group), 2% (medium dose group), and 1% (low dose group) solution, respectively.

Forty-eight hours after the last modeling, the drug was administered percutaneously. Except for the blank and model groups, the model solvent group was smeared with a DMSO solution. The compound calamine lotion group was smeared with compound calamine lotion. The three-dose groups of Zanthoxylum bungeanum and Zanthoxylum schinifolium was smeared with 4%, 2%, and 1% volatile oil solution. 1mL solution was absorbed to cover the whole lesion area, twice a day for 7 days.[24]

Scratching times

After the last modeling or administration, the rats were placed in a transparent observation cage which was the same as the feeding environment. The video camera recorded the scratching behavior of rats in each group within 30 minutes. No one was left in the room during the camera period. At the end of the experiment, the video was played back to count the scratching times of each group. The behavior of rats turning and licking their back or scratching their back with back claws was used to judge the number of scratches. The statistics does not include scratching by front claws.[25] One Way ANOVA and LSD were used for data that obeyed normal distribution and had equal variance between groups, and rank sum test was used for data that did not have equal variance or did not obey normal distribution. $P < 0.05$ indicates that the difference is statistically significant.

Collection of specimens

Twenty-four hours after the last administration, the rats were anesthetized with 1% pentobarbital sodium (300mg/kg). The dorsal skin was taken for HE staining, immunohistochemical staining, Western blot, and QRT-PCR. The dorsal root ganglion (DRG) in the C3-C5 segment spine was then removed for QRT-PCR.[26]

Hematoxylin and eosin stain (HE) staining

The fixed skin tissue for dehydration, wax dipping, embedding, slicing, spreading, baking, and hematoxylin-eosin staining was then photographed under a microscope and collected by the Leica Application Suite image system.[27]

Immunohistochemical staining

The fixed rat skin tissue was taken for dehydration, wax dipping, embedding, slicing, spreading, baking, primary antibody incubation, secondary antibody incubation, DAB staining, hematoxylin staining, and sealing. The distribution of positive substances was then observed under a 200x microscope. The ImageJ image analysis system

semi-quantitatively detected the integral optical density (IOD) value of positive substance to express the contents of PAR-2, TRPV1, and TRPA1.[28,29]

One Way ANOVA and LSD were used for data that obeyed normal distribution and had equal variance between groups, and rank sum test was used for data that did not have equal variance or did not obey normal distribution. P< 0.05 indicates that the difference is statistically significant.

Western Blot

The tissue was first shredded. The shredded tissue and lysate was added to the homogenate tube in the ratio of 20 mg:200 μL. After homogenization, the tissue was centrifuged at 4°C and 12000 g for 15 minutes. The supernatant was prepared for use.[30,31]

According to Table 1, the corresponding standard amount of protein, PBS solution, and 160mL BCA working solution was added to the eight holes of the enzyme plate, and then the absorbance was measured at 562 nm and the standard curve was drawn. Then, 2mL protein to be tested, 18mL PBS, and 160mL BCA working solution was added to the new hole to determine the absorbance. According to the absorbance of the measured sample, the actual concentration of the sample (unit: mg/mL) was calculated.

12% separation gel and 5% concentrated gel was prepared and the comb inserted. The PAGE gel was put into the electrophoresis tank and sample 20μg protein was put in each hole. A 80V electrophoresis was done for 40 min, then 120V electrophoresis for 50 min, followed by 90V transfer membrane 50 min. After the primary antibody and the second antibody are added, the ECL luminescent liquid was dripped on the membrane, then the membrane was placed on the automatic chemiluminescence analyzer, and the band gray value read by TANON GIS software.

One Way ANOVA and LSD were used for data that obeyed normal distribution and had equal variance between groups, and rank sum test was used for data that did not have equal variance or did not obey normal distribution. P< 0.05 indicates that the difference is statistically significant.

QRT-PCR

The tissue samples were homogenated, and the supernatant was taken after centrifugation. After adding chloroform, the supernatant was obtained by centrifugation. After adding isopropanol, the precipitation was obtained by centrifugation. After adding 75% ethanol, the precipitation was obtained by centrifugation, which then dried at room temperature. After adding DNase/RNase-Free Water, it was put in the refrigerator at -80°C[32,33].

After adding the solution to the PCR tube according to Table 2, it was put on the PCR instrument and kept at 42°C for 60 min, and keep it at 70°C for 15min. Reverse transcription products stored at 4°C.

The amplification system is shown in Table 3. The primer sequence required for QRT-PCR is shown in Table 4. The amplification procedure: the pre-denatured condition was kept at 95°C for 3 min, then cycled 40 times, and the condition was kept at 95°C for 5s, at 56°C for 10s, and at 72°C for 25s. The condition of the dissolution curve is that 65°C gradually increases to 95°C, and the temperature rises 0.5°C every 5 s.

One Way ANOVA and LSD were used for data that obeyed normal distribution and had equal variance between groups, and rank sum test was used for data that did not have equal variance or did not obey normal distribution. P< 0.05 indicates that the difference is statistically significant.

Table 1 Protein quantitative sampling.

Hole number	1	2	3	4	5	6	7	8
Protein quantity	0	2	4	6	8	12	16	20
dd H_20	20	18	16	14	12	8	4	0
Protein concentration	0	0.05	0.1	0.15	0.2	0.3	0.4	0.5

Table 2 Reverse transcription system.

Component	Sampling quantity
Total RNA	500 ng
5 × Prime Script II Buffer	4 μL
10 mM dNTP Mix	2 μL
Oligo DT18 primer	1 μL
Recombinant RNase Inhibitor (40 U/μL)	1 μL
Prime Script II RTase (200 U/μL)	1 μL
dd H_2O(DNase/RNase-Free)	To 20 μL
Total volume:	20 μL

Table 3 Amplification system.

Component	Sampling quantity
SYBR FAST qPCR Master Mix	10 μL
Upstream primer F (10 μM)	0.5 μL
Downstream primer R (10 μM)	0.5 μL
cDNA template	1 μL
dd H_2O(DNase/RNase-Free)	8 μL
Total volume	20 μL

Table 4 Primer sequence

Primer name	Primer sequence	Fragment size (bp)
β-actin	F: 5′-CCCATCTATGAGGGTTACGC-3′ R: 5′-TTTAATGTCACGCACGATTTC-3′	150
GRPR	F: 5′-GTTTATGGGCTTATCATCGTG-3′ R: 5′-CCAGCAGCAGTAGGTCTCC-3′	136
TRPV1	F: 5′-CGAGTGCCGACACCTATC-3′ R: 5′-TTCAAGGGTTCCACGAGA-3′	177
TRPA1	F: 5′-GTATCTCCAATGCCCATTATCC-3′ R: 5′-CTACACACAGGGTGGTTGAGG-3′	132
HIR	F: 5′-GTCCCTGTTTCCGTCTCG-3′ R: 5′-CCCTGGCTCTATGCTTGTGT-3′	218
PAR2	F: 5′-CGGGACCCAACAGTAAAG-3′ R: 5′-CGGGAGAAAGACGGTGG-3′	152

Result

Volatile oil yield

$$\text{Volatile oil yield} = \frac{\text{volatile oil quality (g)}}{\text{Zanthoxylum bungeanum powder quality (g)}} \times 100\%.$$

According to the formula, volatile oil yield of Zanthoxylum bungeanum is 3.34%, while that of Zanthoxylum schinifolium is 2.53%. Zanthoxylum bungeanum has more volatile oil than Zanthoxylum schinifolium. GC-MS analyzed the volatile oil components, and the total ion flow diagram was obtained (See Figure 1 and Figure 2).

As shown in Table 5 and Table 6, 25 and 13 chemical constituents were detected in the volatile oil of Zanthoxylum bungeanum and Zanthoxylum schinifolium, respectively. The main chemical constituent of Zanthoxylum bungeanum is monoterpenoids, accounting for 84% of the tested components. The first five compounds with relative contents from high to low were Xanthoxylin, 1-methylidene-4-prop-1-en-2-ylcyclohexane, sabinene, cineole, and (-)-terpinen-4-ol. The main chemical constituent of Zanthoxylum schinifolium is sesquiterpenoids, accounting for 54% of the tested components. The first five compounds with relative contents from high to low were Estragole, cis-Anethol, Spathulenol, carene, and Germacrene D. The results showed that the volatile oil of both Zanthoxylum bungeanum and Zanthoxylum schinifolium contained carene and linalool, but the other components were different, indicating that there were great differences in content and composition between them. The composition and content of the compounds in Zanthoxylum bungeanum and Zanthoxylum bungeanum are different from those reported by Yang X,[34] indicating that the chemical constituents of volatile oil in Zanthoxylum bungeanum and Zanthoxylum bungeanum are closely related to the origin and variety.

Difference value of scratching times

The difference value of scratching times is the difference between the scratching times after modeling and the scratching times after administration. The greater the difference value of the scratching time, the better the

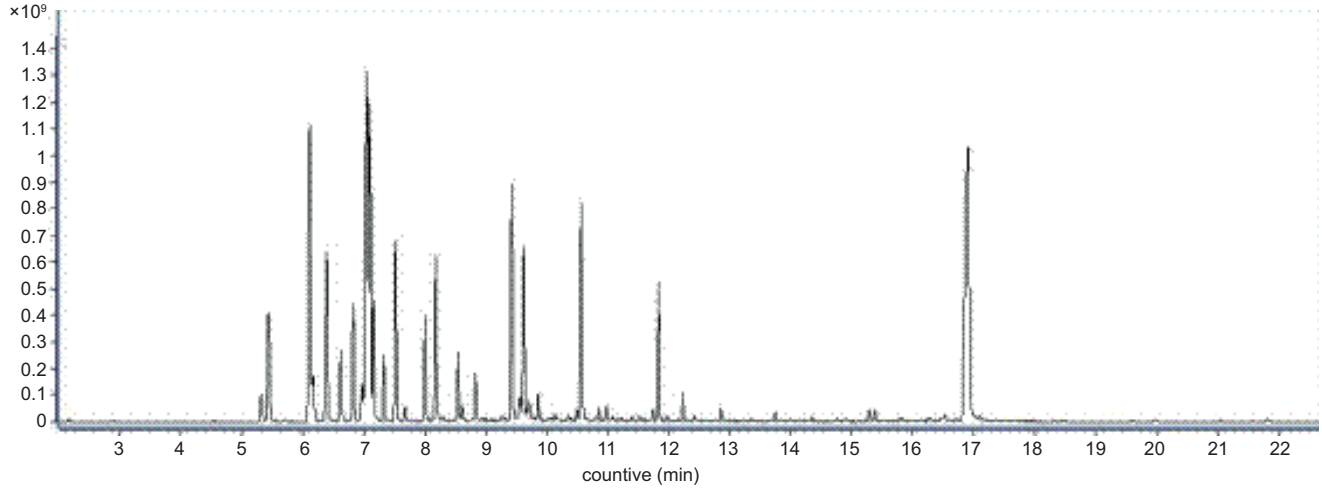

Figure 1 Total ion flow diagram of volatile oil from Zanthoxylum bungeanum.

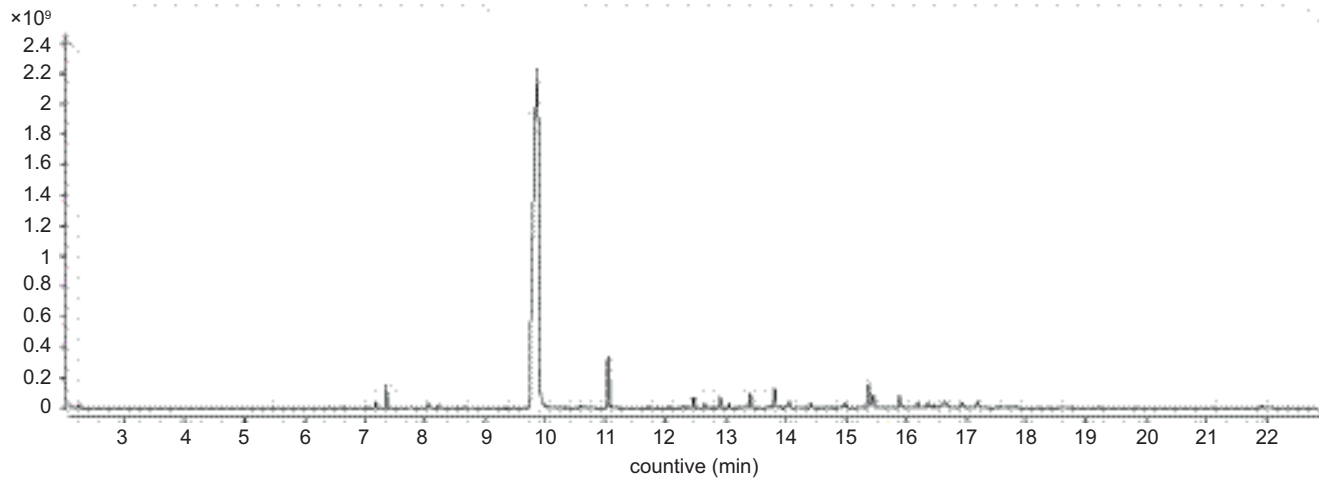

Figure 2 Total ion flow diagram of volatile oil from Zanthoxylum schinifolium.

Table 5 Analysis results of main volatile oil components of Zanthoxylum bungeanum.

Sl. No.	Retention time	CAS Number	Components	Molecular formula	Relative content
1	5.323	2867-05-2	α-thujene	$C_{10}H_{16}$	0.86
2	5.435	13877-91-3	(Z)-β-ocimene	$C_{10}H_{16}$	3.58
3	6.119	3387-41-5	Sabinene	$C_{10}H_{16}$	10.06
4	6.168	99-84-3	β-terpinene	$C_{10}H_{16}$	1.13
5	6.387	18172-67-3	β-Pinene	$C_{10}H_{16}$	4.78
6	6.62	99-83-2	α-phellandrene	$C_{10}H_{16}$	1.81
7	6.825	99-86-5	α-Terpinene	$C_{10}H_{16}$	2.97
8	6.973	527-84-4	o-Cymene	$C_{10}H_{14}$	0.94
9	7.049	499-97-8	1-methylidene-4-prop-1-en-2-ylcyclohexane	$C_{10}H_{16}$	12.61
10	7.102	470-82-6	Cineole	$C_{10}H_{18}O$	8.16
11	7.156	7785-70-8	(1R)-(+)-ALPHA-PINENE	$C_{10}H_{16}$	2.64
12	7.326	13466-78-9	carene	$C_{10}H_{16}$	1.35
13	7.518	99-85-4	γ-Terpinene	$C_{10}H_{16}$	4.02
14	8.001	29050-33-7	δ-4-carene	$C_{10}H_{16}$	2.24
15	8.18	78-70-6	Linalool	$C_{10}H_{18}O$	3.57
16	8.542	29803-82-5	1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol	$C_{10}H_{18}O$	1.4
17	8.828	29803-81-4	1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol	$C_{10}H_{18}O$	1.13
18	9.427	20126-76-5	(-)-Terpinen-4-ol	$C_{10}H_{18}O$	6.6
19	9.62	10482-56-1	(-)-α-Terpineol	$C_{10}H_{18}O$	3.88
20	9.687	16721-38-3	(+/-)-cis-Piperitol	$C_{10}H_{18}O$	0.4
21	9.861	491-04-3	3-methyl-6-propan-2-ylcyclohex-2-en-1-ol	$C_{10}H_{18}O$	0.55
22	10.567	89-81-6	PIPERITONE	$C_{10}H_{16}O$	5.75
23	11.833	80-26-2	α-Terpinyl acetate	$C_{12}H_{20}O_2$	2.78
24	12.231	141-12-8	Neryl acetate	$C_{12}H_{20}O_2$	0.56
25	16.912	90-24-4	Xanthoxylin	$C_{10}H_{12}O_4$	16.25

Table 6 Analysis results of main volatile oil components of Zanthoxylum schinifolium.

Sl. No.	Retention time	CAS Number	Components	Molecular formula	Relative content
1	7.183	4889-83-2	3,6,6-Trimethyl-bicyclo(3.1.1)hept-2-ene	$C_{10}H_{16}$	0.4
2	7.353	13466-78-9	carene	$C_{10}H_{16}$	1.52
3	8.225	78-70-6	Linalool	$C_{10}H_{18}O$	0.28
4	8.690	18368-95-1	1-methyl-4-prop-1-en-2-ylcyclohexa-1,3-diene	$C_{10}H_{14}$	0.29
5	9.879	140-67-0	Estragole	$C_{10}H_{12}O$	84.88
6	11.055	104-46-1	cis-Anethol	$C_{10}H_{12}O$	3.87
7	12.473	515-13-9	beta-elemene	$C_{15}H_{24}$	0.74
8	13.412	6753-98-6	α-Caryophyllene	$C_{15}H_{24}$	1.22
9	13.810	23986-74-5	Germacrene D	$C_{15}H_{24}$	1.48
10	14.413	483-76-1	(+)-DELTA-CADINENE	$C_{15}H_{24}$	0.32
11	15.383	6750-60-3	Spathulenol	$C_{15}H_{24}O$	2.22
12	15.459	1139-30-6	Caryophyllene Oxide	$C_{15}H_{24}O$	1.37
13	16.649	88-84-6	1,4-dimethyl-7-propan-2-ylidene-2,3,4,5,6,8-hexahydro-1H-azulene	$C_{15}H_{24}$	1.43

antipruritic effect of the drug is. Figure 3 showed no statistical difference in the difference value of scratching times between the model and model solvent group ($P > 0.05$), indicating that DMSO has no antipruritic effect. Compared with the model group, the difference value of scratching times in the calamine lotion group, medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium group was significantly higher, which indicated that the antipruritic effect of these three groups was better. Among the three doses of Zanthoxylum bungeanum and Zanthoxylum schinifolium, the difference value of scratching times in the middle dose of Zanthoxylum bungeanum and Zanthoxylum schinifolium was the largest. The results showed that the antipruritic effect of middle dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was the best. Compared to the calamine lotion group, the

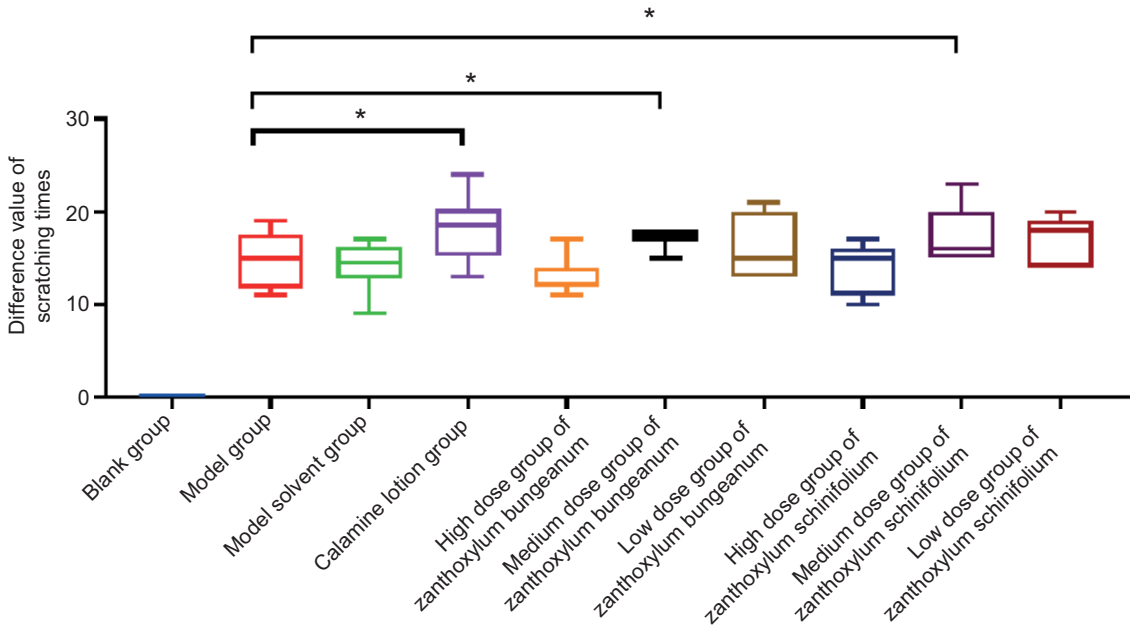

Figure 3 The increase in the number of scratches after successful modeling.

difference value of scratching times of middle dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was slightly reduced, and the difference was not statistically significant ($P > 0.05$). The results showed that the antipruritic effect of the calamine lotion group, middle dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was the same. Having a larger dataset could provide further insight on this.

Observation of pathomorphological changes by HE staining

As shown in Figure 4, the structure of the epidermis and dermis in the blank group is intact, and the thickness is average. Hyperkeratosis and acanthosis were found in the model group and the model solvent group. Severe edema of dermis and infiltration of lymphocytes and eosinophils can be seen. The results showed that DMSO had no therapeutic effect.

Compared with the model group, the hyperkeratosis, edema, and infiltration in the calamine lotion group, the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium were significantly lower, which indicated that the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium could significantly improve the pathological morphology.

When compared with the blank group, the skin lesions in the model group were severe, and when compared with the model group, the calamine lotion group, medium-dose group of Zanthoxylum bungeanum, and Zanthoxylum schinifolium had a remarkable effect on improving the case.

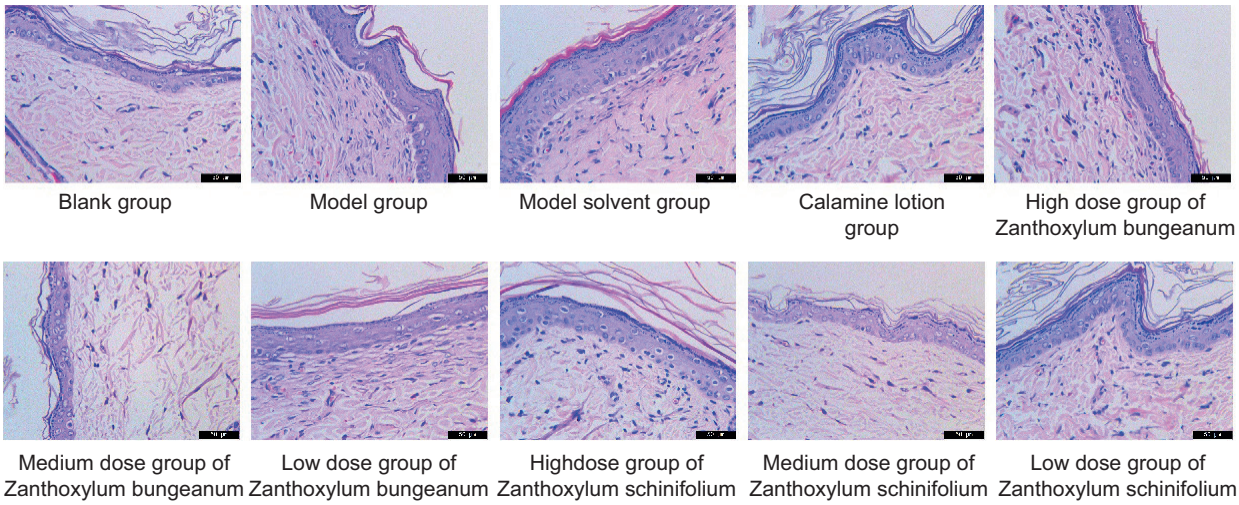

Figure 4 HE staining of back skin tissue (×400, scale label=50 μm).

The expressions of H1R, PAR-2, TRPV1, and TRPA1 detected by Immunohistochemical staining

As shown in Figure 5-9, the contents of H1R, PAR-2, and TRPV1 in the model group were significantly higher than those in the blank group, indicating that chronic eczema may be related to the up-regulation of the expression of H1R, PAR-2, and TRPV1. Compared with the model group, the contents of H1R, PAR-2, and TRPV1 in the calamine lotion group, medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium decreased significantly, indicating that the antipruritic effect of medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium may be related to the down-regulation of the expression of H1R, PAR-2, and TRPV1. Among the three dose groups of Zanthoxylum bungeanum and Zanthoxylum schinifolium, the down-regulation effect of the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium were the most significant. Compared with the calamine lotion group, the expression of H1R, PAR-2, and TRPV1 in the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium changed slightly, but the difference was not statistically significant.

Compared with the blank group, TRPA1 in the model group was significantly up-regulated ($P < 0.05$). Compared to the model group, the TRPA1 of the calamine lotion group and high dose group of Zanthoxylum schinifolium was significantly down-regulated, while the TRPA1 of the medium dose group of Zanthoxylum bungeanum was significantly up-regulated,

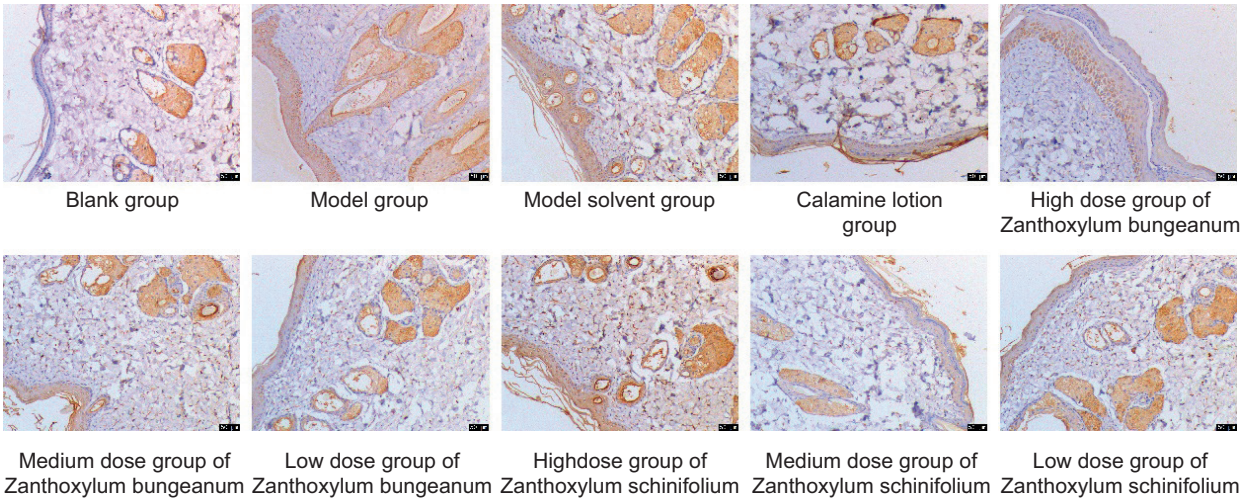

Figure 5 Expression of H1R in skin tissue (×200, scale label = 50 μm). Yellow indicates the expression of H1R. Compared with the blank group, the expression of H1R in the model group increased significantly. Conversely, compared with the model group, the expression of H1R in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium decreased significantly.

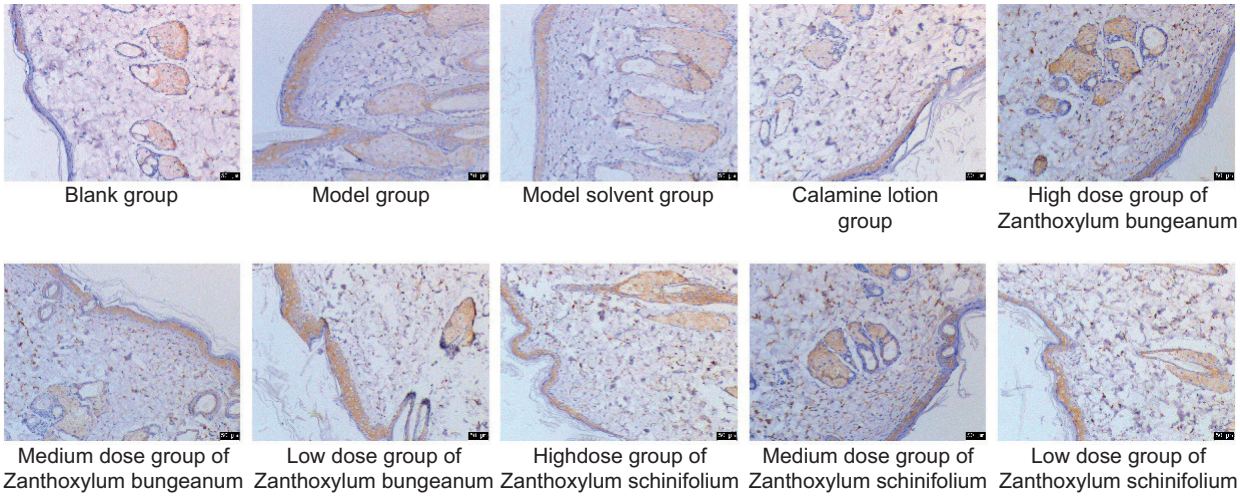

Figure 6 Expression of PAR-2 in skin tissue (×200, scale label=50 μm). Yellow indicates the expression of PAR-2. Compared with the blank group, the expression of PAR-2 in the model group increased significantly. Conversely, compared with the model group, the expression of PAR-2 in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum, and Zanthoxylum schinifolium decreased significantly.

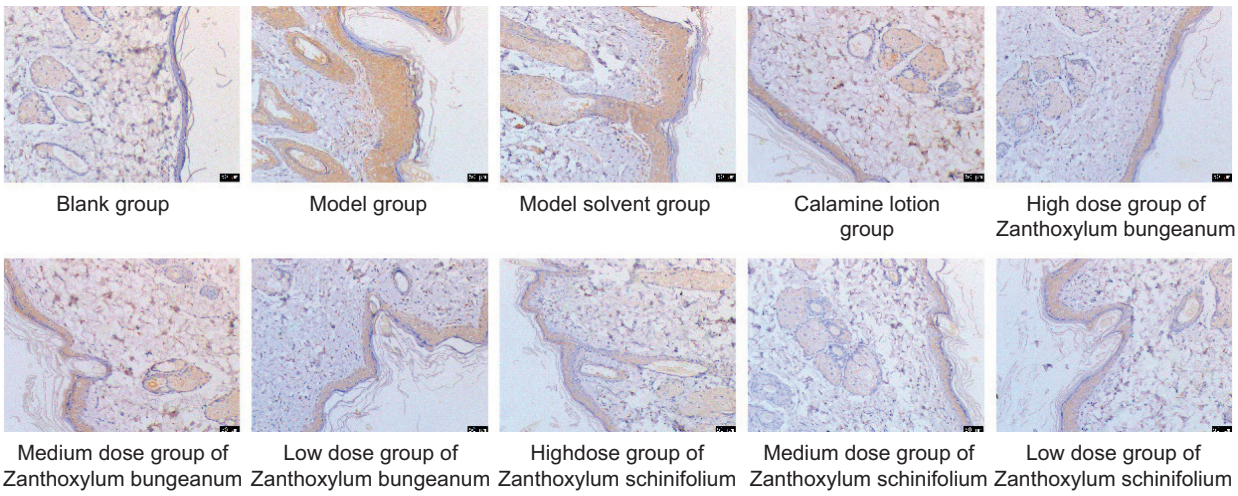

Figure 7 Expression of TRPV1 in skin tissue (×200, scale label=50 μm). Yellow indicates the expression of TRPV1. Compared with the blank group, the expression of TRPV1 in the model group increased significantly. Conversely, compared with the model group, the expression of TRPV1 in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium decreased significantly.

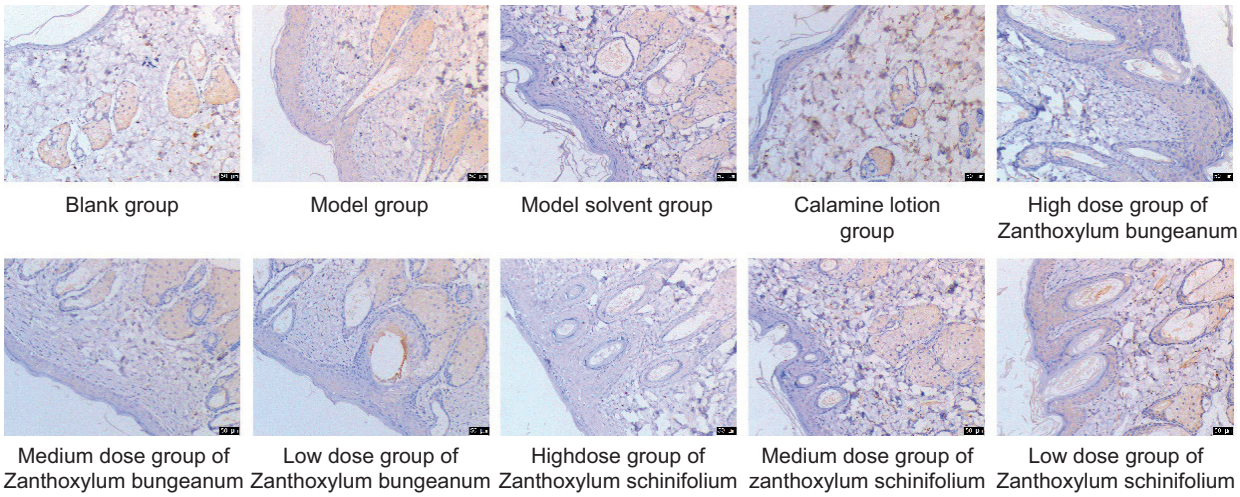

Figure 8 Expression of TRPA1 in skin tissue (×200, scale label=50 μm). Yellow indicates the expression of TRPA1. Compared with the blank group, the expression of TRPA1 in the model group increased significantly. However, compared with the model group, there was no significant difference between Zanthoxylum bungeanum group and Zanthoxylum schinifolium group.

indicating that the antipruritic effect of Zanthoxylum schinifolium may be related to the down-regulation of expression of TRPA1, while Zanthoxylum bungeanum may up-regulate expression of TRPA1, affecting the antipruritic effect.

Compared with the blank group, the expression of H1R in the model group was up-regulated. Compared with the model group, the expression of H1R in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was significantly down-regulated; Compared with the blank group, the expression of PAR-2 in the model group was up-regulated. In comparison with the model group, the expression of PAR-2 in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was significantly down-regulated.

Compared with the blank group, the expression of TRPV1 in the model group was up-regulated, and with the model group, the expression of TRPV1 in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was significantly down-regulated. Compared with the blank group, the expression of TRPA1 in the model group was up-regulated. Compared with the model group, there was no significant difference in the expression of TRPA1 with the medium-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium.

The expressions of H1R, PAR-2, TRPV1, and TRPA1 detected by Western blot

As shown in Figure 10, 11, the expressions of H1R, PAR-2 and TRPV1 in the model group were significantly higher than those in the blank group. Compared with the model group,

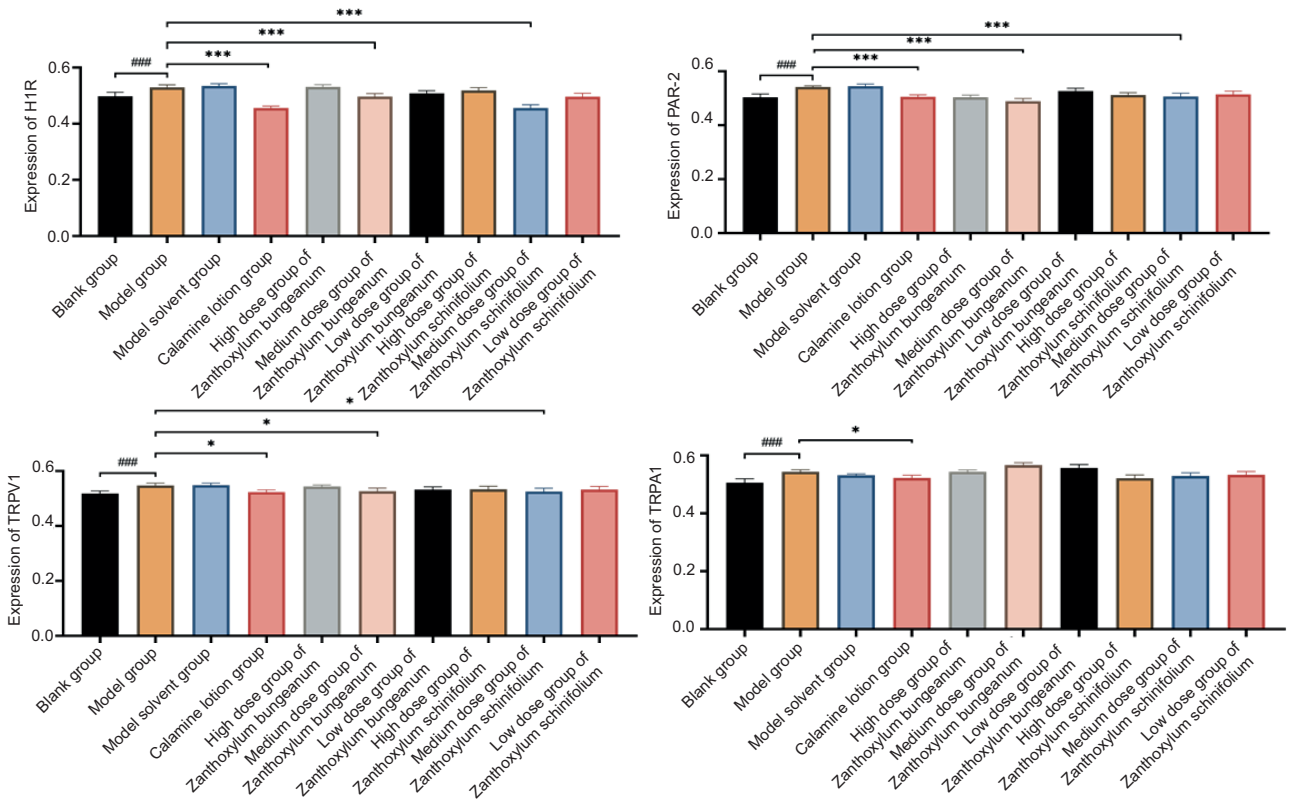

Figure 9 The expression of H1R, PAR-2, TRPV1, TRPA1.

the expressions of H1R, PAR-2, and TRPV1 in the calamine lotion group, medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium were significantly decreased, which indicated that the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium might play the antipruritic effect by down-regulating the expression of H1R, PAR-2, and TRPV1. Among the three doses of Zanthoxylum bungeanum and Zanthoxylum schinifolium, the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium decreased the expression of H1R, PAR-2, and TRPV1 most significantly. The expression of H1R, PAR-2, and TRPV1 in medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium had no significant difference compared with calamine lotion group, which indicated that the calamine lotion group, medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium had the same effect on down-regulating the expression of H1R, PAR-2 and TRPV1.

Compared with the blank group, the expression of TRPA1 in model group and model solvent group had no significant change, but the expression of TRPA1 in three dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium were significantly up-regulated. It shows that the chronic eczema model was not associated with expression of TRPA1, and Zanthoxylum bungeanum and Zanthoxylum schinifolium can up-regulate the expression of TRPA1 and affect the antipruritic effect.

Compared with the blank group, the expression of H1R in the model group was upregulated. Compared with the model group, the expression of H1R in the calamine lotion group, the medium-dose group of Zanthoxylum bungeanum, and Zanthoxylum schinifolium was significantly down-regulated. Compared with the blank group, the expression of PAR-2 in the model group was up-regulated. Compared with the model group, the expression of PAR-2 in the calamine lotion group, high-dose group of Zanthoxylum bungeanum, and the low-dose group of Zanthoxylum schinifolium was significantly down-regulated; when compared with the blank group, the expression of TRPV1 in the model group was up-regulated. Compared with the model group, the expression of TRPV1 in the high-dose group of Zanthoxylum bungeanum was significantly down-regulated. Compared with the blank group, the expression of TRPV1 in the model group had no significant change.

The expression of H1R, PAR-2, TRPV1, TRPA1, and GRPR mRNA detected by QRT-PCR

As shown in Figure 12, compared with the blank group, the expression of H1R, PAR-2, TRPV1, and GRPR mRNA in the model group were significantly higher, indicating that chronic eczema may up-regulate the expression of H1R, PAR-2, TRPV1, and GRPR. Compared with the model group, the expression of H1R, PAR-2, TRPV1, and GRPR mRNA in calamine lotion group, medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium decreased significantly, which indicated that the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium had antipruritic effect by down-regulating the expression of H1R, PAR-2, TRPV1 and

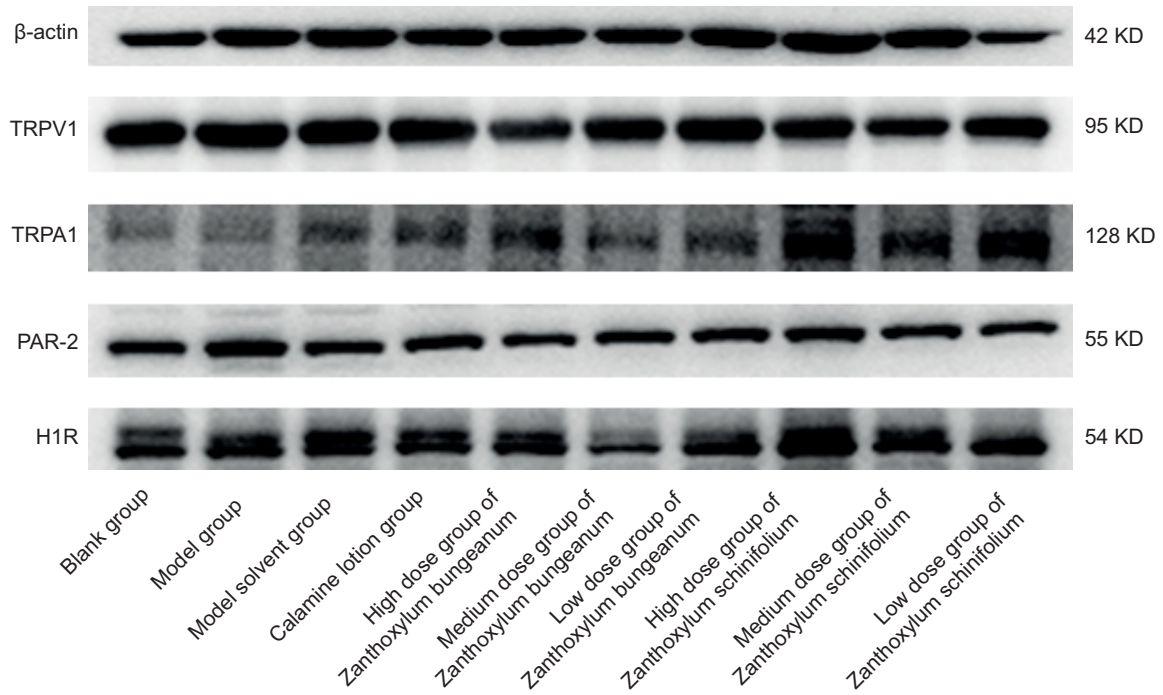

Figure 10 Protein expression.

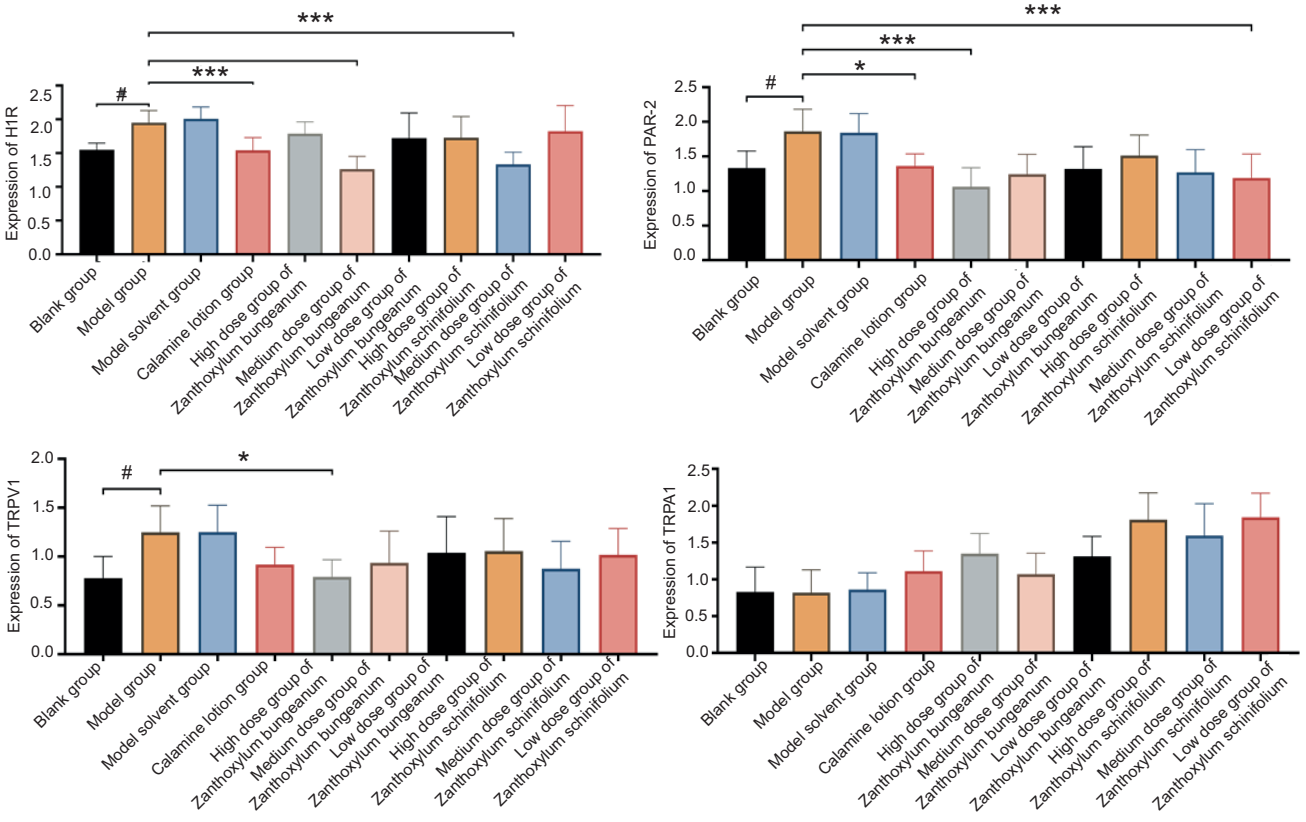

Figure 11 The relative protein expression levels of H1R, PAR-2, TRPV1 and TRPA1.

GRPR. Among the three dose groups of Zanthoxylum bungeanum and Zanthoxylum schinifolium, the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium down-regulated the expression of H1R, PAR-2, TRPV1, and GRPR most significantly. Compared with the calamine lotion group, the expression of H1R, PAR-2, and TRPV1 mRNA in the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium decreased, while the expression of GRPR mRNA increased. There was no significant difference in the expression of H1R, PAR-2, TRPV1, and GRPR mRNA

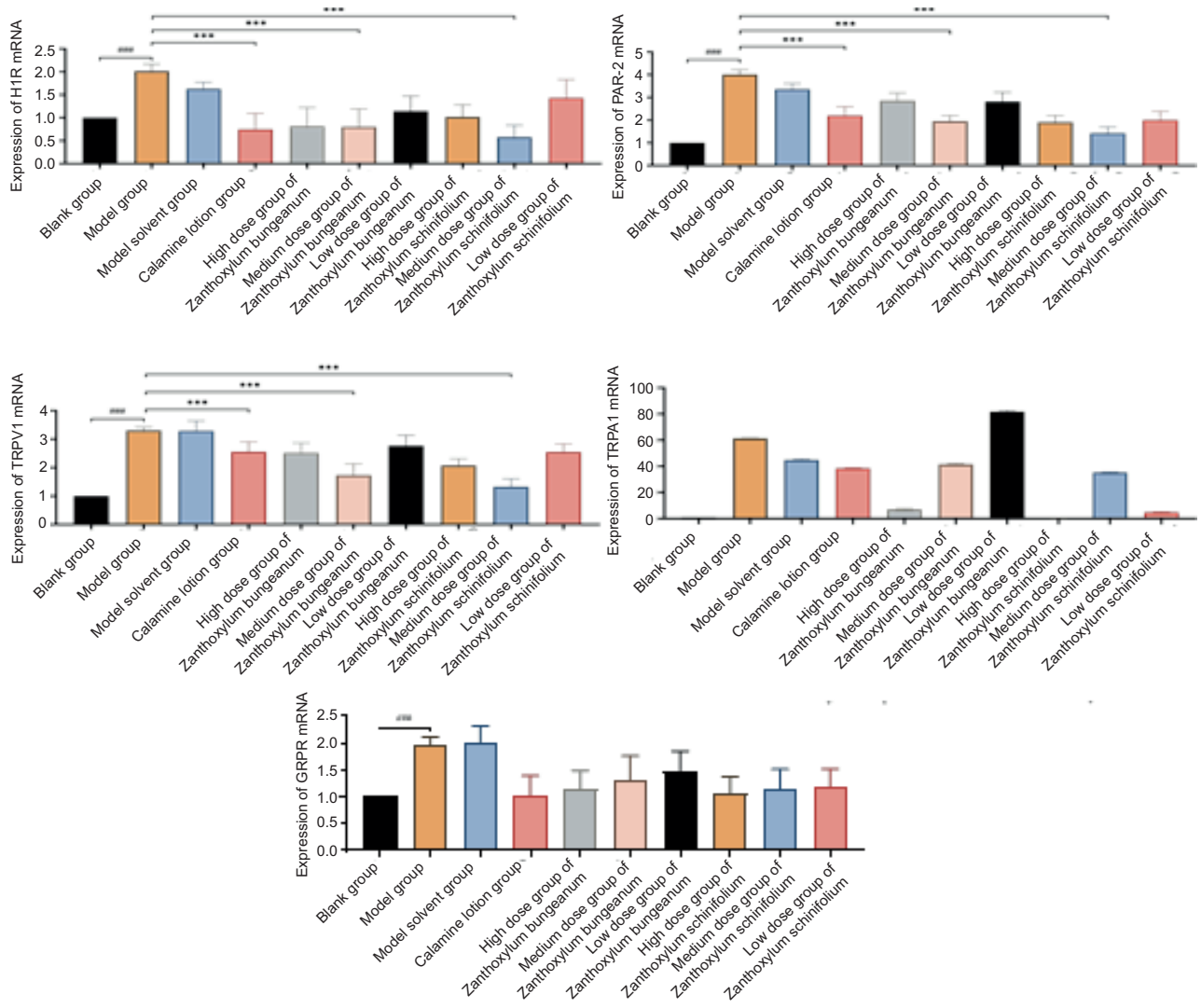

Figure 12 Relative expression level of mRNA.

between the medium dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium, indicating that the two groups had the same effect on down-regulating the expression of H1R, PAR-2, TRPV1 and GRPR.

Compared with the blank group, the expression of TRPA1 in the model group and the model solvent group increased significantly, indicating that chronic eczema may up-regulate the expression of TRPA1. Compared with the model group, the calamine lotion group, the high dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium significantly down-regulate the expression of TRPA1, indicating that high dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium may have antipruritic effect by down-regulating the expression of TRPA1. Compared with the calamine lotion group, the expression of TRPA1 in the high dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium was significantly lower.

Compared with the blank group, the expression of H1R in the model group was significantly up-regulated, and compared with the model group, the expression of H1R in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum, and Zanthoxylum schinifolium were significantly down-regulated. Compared with the blank group, the expression of PAR-2 in the model group was significantly up-regulated. Compared with the model group, the expression of PAR-2 in the calamine lotion group, the medium-dose group of Zanthoxylum bungeanum, and Zanthoxylum schinifolium were significantly down-regulated. Compared with the blank group, the expression of TRPV1 in the model group was significantly up-regulated. In comparison with the model group, the expression of TRPV1 in the calamine lotion group, medium-dose group of Zanthoxylum bungeanum, and Zanthoxylum schinifolium were significantly down-regulated. Compared with the blank group, the expression of TRPA1 in the model group was significantly up-regulated, and with the model group, the expression of TRPA1 in the calamine lotion group, high-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium were significantly down-regulated. Compared with the blank group, the expression of GRPR in the model group was significantly up-regulated. In comparison with the model group, the expression of GRPR in the calamine lotion group, the high-dose group of Zanthoxylum bungeanum and Zanthoxylum schinifolium were significantly down-regulated.

Discussion

A cross-sectional study conducted in China in 2014 reported the prevalence of AD in adults to be 7.8%, suggesting that adult-onset AD was fairly common in this population.[35] Atopic dermatitis has a significant and increasing prevalence in both pediatric and adult population worldwide.[3]

Chinese medicine proves that volatile oil of Zanthoxylum bungeanum and Zanthoxylum schinifolium can treat atopic dermatitis. Some people have clinically verified the efficacy of volatile oil, but no one has studied the mechanism of its treatment of atopic dermatitis, and no one has studied whether the efficacy of volatile oil in improving skin lesions or suppresses itching or both. At present, we have experimentally demonstrated the anti-itch mechanism of volatile oil, and are in the process of designing experiments to verify whether the volatile oil can improve skin lesions. The research will fill this blank area in an effort to provide a safe and effective topical treatment drug for patients with atopic dermatitis.

GC-MS, scratching times, HE staining, immunohistochemical staining, Western Blot, and QRT-PCR was used to observe the antipruritic effect and antipruritic mechanism of Zanthoxylum bungeanum and Zanthoxylum schinifolium volatile oil on chronic eczema model. It was found that Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil can effectively reduce the scratching times, Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil can down-regulate the expression of H1R, PAR-2, TRPV1, and GRPR, indicating that the mechanism of relieving itching may be through the intervention of H1R, PAR-2-TRPV1-GRPR pathway. Although there are differences in the content and composition of volatile oil between Zanthoxylum bungeanum and Zanthoxylum schinifolium, the curative effect is the same. This may be because the effects of the two groups of active ingredients on the expression of each target site add up to be about equal, although the two may have different active ingredients.

There are many limitations in this experiment, which need to be further studied. The experiment lacks reverse verification, such as knocking out target genes or using receptor inhibitors, and then studying the antipruritic mechanism. The experiment did not clarify the specific effective ingredients of Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil in relieving itching, which needs to be further studied. There are significant differences in the composition of volatile oil between Zanthoxylum bungeanum and Zanthoxylum schinifolium, but there is no significant difference in the antipruritic effect between them, which needs to be further studied. The therapeutic effects of Zanthoxylum bungeanum oil and Zanthoxylum schinifolium oil do not show a positive dose-effect relationship, which needs to explored further.

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