Compound Traditional Chinese Medicine Dermatitis Ointment Ameliorates Inflammatory Responses and Dysregulation of Itch-Related Molecules in AD

Rongrong Zhang

Changchun University of Chinese Medicine

Shuai Shao

Changchun University of Chinese Medicine

Yingxin Shen

Changchun University of Chinese Medicine

Jiaming Sun

Changchun University of Chinese Medicine

Songlan Piao

Changchun University of Chinese Medicine

Daqing Zhao

Changchun University of Chinese Medicine

Guangzhe Li

Changchun University of Chinese Medicine

mingming yan (✉ 386759102@qq.com)

Changchun University of Chinese Medicine <https://orcid.org/0000-0001-8782-5854>

Research Article

Keywords: MS, Medicinal substance, Atopic dermatitis, MAPK

Posted Date: September 3rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-783506/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease accompanied by an itchy and scaly rash. Compound traditional Chinese medicine dermatitis ointment (CTCMDO) is a traditional classics aimed at AD composed of a mixture of extracts from five plants known to have anti-inflammatory and antiallergic effects.

Materials and methods: In this study, we used HPLC and LC/MS to analyze the effective components of CTCMDO in detail and establish its HPLC fingerprint analysis. On this basis, this article studied the anti-inflammatory and antipruritic activities of CTCMDO in the treatment of DNCB-induced AD in mice.

Results: Through comparison with literature data, a total of 43 compounds were identified, including phenylpropionic acid compounds; alkaloid compounds; curcumin compounds and lignans. On this basis, a fingerprint with 17 common peaks was established. In AD-like mice, CTCMDO treatment suppressed the scratching behavior induced by DNCB in a dose-dependent manner and inhibited the production of Th1/2 cytokines in serum. CTCMDO treatment reversed the up regulation of P substance levels of itch-related genes in the skin. Furthermore, CTCMDO suppressed the phosphorylation of JNK/ERK and p38 in the skin.

Conclusion: In all, our work indicated that CTCMDO can significantly attenuate the pathological alterations of Th1/2 cytokines and itch-related mediators, and inhibit the phosphorylation of MAPKs to treat AD.

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease and often causes other allergic disorders, such as food allergies, asthma, and rhinitis. As a serious global public health problem, the morbidity of AD has increased in recent years (Torres T, *et al.*, 2019). AD is a disease arising from complex interaction of genetic factors with skin barrier dysfunction and environmental factors (Williams and Flohr, 2006; Leung *et al.*, 2013). The skin lesions are often on their faces and the extensor surfaces of the limbs. Although AD is not life-threatening, frequent complications such as itchiness, sleep deprivation, social embarrassment, and depression because of skin lesions on body surface would substantially affect the physical and mental health of the patients. (De Benedetto *et al.*, 2012; Leung *et al.*, 2013)

Current research on AD pathogenesis suggests that AD is a well-established inflammatory disease involving dysregulation of both innate and adaptive immunity. In particular, AD is mainly a dysregulation of the T-cell mediated adaptive immunity system (Weidinger S *et al.*, 2016). Abnormal secretion of T-helper (Th) 1 and Th2 cytokines is considered as one of the main causes of AD, especially interleukin (IL)-4 and IL-6 are the triggers of AD (David Boothe W, *et al.*, 2017), which can promote the proliferation of B and T cells, synthesis of (immunoglobulin) IgE, induction of Th2 differentiation. Current strategies for AD treatment include topical application of emollients, corticosteroids and calcineurin inhibitors, phototherapy, oral administration of steroids and immunosuppressants, and biologics (Guttman-Yassky

E, *et al.*, 2017). However, these treatment modalities usually come with some side effects such as skin atrophy and development of drug tolerance. As a form of complementary and alternative medicine, traditional Chinese medicine (TCM) has been applied for treating various skin diseases for a long time (Cao *et al.*, 2018; Guo *et al.*, 2017; Yu *et al.*, 2018).

In this study, we introduce Compound traditional Chinese medicine dermatitis ointment (CTCMDO), which is a mixture of oil extracts from six plants (*Coptis chinensis*, *Phellodendron*, *Angelica*, *Rehmannia glutinosa*, *Curcuma* and Sesame oil), as a novel medication for treating AD. Each traditional Chinese medicine in CTCMDO has been shown to demonstrate anti-inflammatory and antiallergic activity. As CTCMD is the most prescribed drug in dermatology in our hospital, it has been clinically used to more than 85% outpatient for the treatment of AD, rash and many other inflammatory skin diseases. Although the efficacy of CTCMDO has been widely recognized by doctors and patients, there is a lack of research on its anti-AD pharmacology and underlying mechanism. In this study, we used AD mice model to systematically analyze the anti-inflammatory and anti-allergy mechanism of CTCMDO and its regulation on the NF- κ B/MAPK signaling pathways.

1. Materials And Methods

1.1 Herb materials

Coptis chinensis (*Coptis chinensis* Franch, huanglian, No.20180301), *Phellodendron* [*Phellodendron amurense* Rupr, huangbai, No.20180101], *Angelica* (*Angelica archangelica* L, danggui, No.20180601), *Rehmannia glutinosa* (*Rehmannia glutinosa*, dihuang, No.c176181205), *Curcuma* (*Curcuma aeruginosa* Roxb, jianghuang, No.20180501) and Sesame oil (*Sesamum indicum* L, No.20180101) were bought from Jilin Xiancao Medicine Co., Ltd. Identification and authentication of the herb materials were carried out at Changchun University of Chinese Medicine. voucher specimens were deposited at the Jilin Ginseng Academy, Changchun University of Chinese Medicine, Jilin, China.

1.2 Chemicals and reagents

The following antibodies, Rabbit Anti-ERK1+ERK2 antibody (bs-0022R), Rabbit Anti-phospho-ERK1/2 antibody (bs-3016R), Rabbit Anti-P38 MAPK antibody (bs-0637R), Rabbit Anti-Phospho-P38 MAPK (Thr180+Tyr182) antibody (bs-0636R), Rabbit Anti-JNK1+JNK2+JNK3 (bs-2592R) and Rabbit Anti-phospho-JNK1+JNK2+JNK3 (T183+T183+T221) antibody (bs-4163R) antibody were used in this study. Compound dexamethasone acetate cream was obtained from Guangzhou Baiyunshan Pharmaceutical Group Co., Ltd (Guangzhou, China). Curcumin, Sesamin, Berberine, Coptisine hydrochloride, Phellodendrine chloride, Ferulic acid standards with the purity greater than 98% were obtained from China National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Assay kits for IgE, Interleukin (IL)-4, tumor necrosis factor (TNF)- α , interleukin (IL)-6, Interleukin (IL)-2 and Interferon (INF)- γ were purchased from RD, USA.

1.3 Instrument

Polypropylene centrifuge tube (Wuhan Saiweier Biological Technology Co. Ltd.), speed micro refrigerated centrifuge (D3024R-Beijing Dalong Xingchuang Experimental Instrument Co. Ltd.), vortex oscillator (MX-F-Wuhan Saiweier Biological Technology Co. Ltd.) , Multi-tube vortex mixer (MTV-100-Hangzhou Aosheng Instrument Co. Ltd.), ultrasonic cleaner (JP-040S-Shenzhen Jiemeng Cleaning Equipment Co. Ltd.), pipette (Eppendorf), chromatograph (UltiMate 3000 RS -Thermo Fisher Technology (China) Co. Ltd.), Agilent 1260 High Performance Liquid Chromatograph (Agilent, USA), Q Exactive High Resolution Mass Spectrometer (Thermo Fisher Technology (China) Co. Ltd.), UV Spectrophotometer (UV-1700-Shimadzu, Japan) ALB-2.24 million analytical balance (Sartorius Scientific Instruments Co. Ltd.); METTLER-AE240 electronic analytical balance (METTLER, Switzerland); DT5-2B low-speed desktop centrifuge (Beijing Times Beili Centrifuge Co. Ltd.); DZF-6050 vacuum drying oven (Shanghai Yiheng Scientific Instrument Co. Ltd.); HH-6 digital display constant temperature water bath (Jintan Jiamei Instrument Co. Ltd.); 302A temperature and humidity control box (Shanghai Shuoguang Electronic Technology Co. Ltd.); enzyme Marker (Meigu Molecular Instruments (Shanghai) Co. Ltd.); Semi-automatic tissue dehydration machine (Leica, Germany); Fully automatic tissue embedding machine (Leica, Germany); Semi-automatic rotary microtome (Leica, Germany); Spreader (Leica, Germany)); Dryer (Leica, Germany); Photographic optical microscope (Olympus, Japan).

1.4 animals

BALB/c female mice (18-22g) were purchased from the Hongda Biotechnology Co., Ltd, Changchun, Jilin, China (Certificate: No.SCXK(Liao)2015-0001) and acclimatized for 7 days. Mice were kept in controlled ambient temperature ($24 \pm 2^{\circ}\text{C}$) and humidity ($60 \pm 10\%$) under 12 hours light-dark cycles; water was available ad libitum. The animal protocol was approved by the Animal Care Ethics Committee of Changchun University of Chinese Medicine (Changchun, Jilin, China, Approval No. 20181011).

2 Methods

2.1 Identification of components of CTCMDO

2.1.1 Sample processing

Precisely weigh 12g of CTCMDO, wrap it with filter paper, and place it in a Soxhlet extractor. Add appropriate amount of petroleum ether and heat until the petroleum ether is colorless. After discarding the petroleum ether, add an appropriate amount of methanol solution and reflux until the methanol is colorless. Pour out the extract, evaporate the solvent, add 2 ml of methanol to dissolve, filter through a $0.22 \mu\text{m}$ microporous membrane, and take the additional filtrate to obtain.

2.1.2 Chromatographic and Mass spectrometry conditions

Chromatographic analysis was performed in an UltiMate 3000 RS system equipped with a quaternary pump, vacuum degasser, autosampler and diode array detector. The mobile phase is 1% chromatographic formic acid aqueous solution (A)-0.1% formic acid acetonitrile (B). The detailed mobile

phase procedure is shown in Table 1; the chromatographic column uses a C18 column (150×2.1mm 1.8μm) with a temperature of 35°C; The injection volume is 10μl, and the flow rate is 0.3ml/min.

The Q Exactive high-resolution mass spectrometer equipped with an electrospray ionization source was used in the positive ion mode and the negative ion mode, controlled by the Thermo Fisher software system. Select the automatic MS2 mode of the mass spectrometer to analyze the sample. Use the following operating parameters: Spray Voltage: 3.8kV (Positive), Capillary Temperature: 300°C, Collision gas: High purity argon (purity ≥99.999%) Sheath gas: Nitrogen (purity ≥99.999%), 40Arb, auxiliary gas (Aux gas heater temp): nitrogen (purity ≥99.999%), 350°C data collection time: 30.0min scan range: 150.0–2000.0m/z. The recorded data is processed by the applied Thermo Xcalibur software system.

2.1.3 Establishment of CTCMDO fingerprint

High performance liquid chromatography (HPLC) analysis for CTCMDO sample solution was performed on Agilent 1260 Infinity HPLC system equipped with a UV detector. Chromatographic separation was conducted on Sepax Bio-C18 column (4.6×250mm, 5μm, from Sepax Technologies, Delaware, USA). The solvent system composed of solvent A (acetonitrile) and solvent B (0.1% phosphoric acid in water) in the following gradient (Table 2). Operating conditions were as follows: detection wavelength, 278nm; flow rate, 1.0ml/min; column temperature, 30°C; injection volume, 20μl. 14 batches of CTCMDO were prepared and measured separately to identify phytochemical constituents.

2.2 Docking Procedure.

Molecular docking is a method of placing the ligand in the binding area of the receptor through computer simulation and calculating its physical and chemical parameters to predict the binding affinity and conformation of the two. The System Dock Web Site server (<http://systemsdock.unit.oist.jp/iddp/home/index>) evaluates the ligand-receptor binding potential through the molecular docking function of the Docking score. The four targets with the highest degree value were selected, and they were imported into the System Dock Web Site server, and molecular docked with the active ingredients of CTCMDO. The results were obtained and evaluated by analyzing the value of Docking Score (Xing Lv *et al.*, 2020, Yanjiao Qi *et al.*, 2021).

2.3 Experimental validation

2.3.1 Preparation of CTCMDO

Take Coptischinensis, Phellodendron, Angelica, Rehmanniaglutinosa and Curcuma by prescription Put them in 360g sesame oil, fry at 120°C for 45min, Hot filtration, get medicated oil (The medicinal oil will be used for composition analysis), add beeswax at a ratio of 3:1 (medicinal: beeswax) and keep stirring until the beeswax is completely dissolved, After all the beeswax is dissolved, stop heating and keep stirring to room temperature and get CTCMDO.

2.3.2 Experimental procedures

Mice (n=10) were divided into 6 groups: NOR (blank group), DNCB (DNCB sensitized group as negative control group), and DEX (compound dexamethasone acetate treated group as positive control group), LOW (low dose group, Specifications of CTCMDO: 75mg/g, crude drug/ointment), MED (medium dose group, Specifications of CTCMDO: 150mg/g, crude drug/ointment), HIGH (high dose group, Specifications of CTCMDO: 75mg/g, crude drug/ointment).

The hair in the area of the back skin was shaved 1 day before the experiment, and the shaved back skin was treated with an application of 100 μ L 1% DNCB solution (dissolved in a 4:1 mixture of acetone and olive oil) for sensitization (for 3 days). The same volume of acetone/olive oil carrier was applied to the blank group. After the first induction, the mice were housed without any further treatment (for 4 days), in the secondary sensitization, 100 μ L of 0.5%DNCB solution was applied to the ear skin. The positive control group and the drug-administered group were treated with DEX and CTCMDO 4 hours before the second sensitization of DNCB, while the blank group and the model group were treated with blank matrix.

2.3.3 Determination of ear swelling

After stopping the drug, the mice were sacrificed, the left and right ears (about 8 mm) were removed with a puncher and quickly weighed on the analytical balance, and the difference in weight between the ears was used as the swelling degree.

2.3.4 ELISA detects the levels of cytokines (INF- γ , TNF- α , IL-6, IL-2, IL-4, IgE)

The total serum samples (n=5) were collected at 24h after DNCB application on day 19, and the level of INF- γ , TNF- α , IL-6, IL-4, IgE and IL-2 in serum were detected by the mouse Enzyme-Linked Immunosorbent Assay (ELISA) Kits. ELISA was performed according to the manufacturer's instruction and quantitation was done with ELISA reader using 450nm filters. Cytokine concentrations were calculated using a linear regression equation obtained from standard absorbance values.

2.3.5 Histological analysis

After fixing the back skin in 10% formalin for 24 hours, it was dehydrated and then embedded in paraffin and sliced (4 microns thick). The sections of back skin were stained with Hematoxylin and Eosin (H&E) and observed by optical microscopy. Thicknesses of the epidermis, dermis, and the number of inflammatory cells were measured using Leica Application Suite. The magnification was $\times 200$.

2.3.6 Immunohistochemistry

The skin sections for immunohistochemistry were carried out in the same manner as histological analysis. After the deparaffinization and rehydration, the skin sections were boiled in 10 mM sodium citrate buffer for antigen retrieval. Slides were treated with 3% H₂O₂ for 30 min to reduce endogenous peroxidase and with normal goat serum (in PBS with 5% NHS, 5% fetal bovine serum, 2% bovine serum albumin (BSA), 0.1% Triton X-100) to minimize nonspecific binding. After blocking, the sections were

incubated with antibodies against substance P overnight at 4°C. After washing with PBS, the sections were incubated with secondary anti-goat antibody for 1 h at RT. Sections were then stained using the Avidin/Biotinylated Enzyme Complex (ABC) Kit and the substrate chromogen mixture was prepared immediately before use. Immunoreactivity was viewed with an LAS. The magnification was $\times 200$.

2.3.7qRT-PCR method to detect the mRNA expression of p-p38 and p38 protein in mouse skin tissue

After administration for 11 days, the expression of p38MAPK mRNA in mouse skin tissue was detected, and the total RNA of mouse skin tissue was extracted by Trizol method for RT-PCR amplification, p38MAPK upstream primer 5'-GATTGAGAT-GATTTTGGAG-3', downstream primer 5'-GTATGTTAAG - TATATGATTG-3', the amplified fragment is 482bp; PCR reaction conditions: 45 s at 94 °C, 50 s at 55 °C, 75 s at 72 °C, after 40 cycles to obtain p38MAPKmRNA cycle threshold (Ct), use $\Delta\Delta C_t$ ($\Delta\Delta C_t = C_t \text{ target gene} - \Delta C_t \text{ internal reference gene}$) analysis, calculate the relative expression of $2^{-\Delta\Delta C_t}$ target gene.

2.3.8 Western Blot analysis

Take the weighed skin tissues of each group of mice, cut them into small pieces with scissors, and extract the protein from the tissues according to the kit instructions to obtain the total protein of each group. Use the BCA kit to determine the protein concentration. Use 10% SDS-PAGE gel electrophoresis transfer membrane, put PVDF membrane into 5% skimmed milk powder blocking solution for 1 h, wash the membrane 3 times with TBST, 5 min each time; add diluted primary antibody (NF- κ B, ERK1 /2, phospho-ERK1/2, JNK, phospho-JNK, p38, and phospho-p38 primary antibodies diluted 1:1000) Incubate at room temperature for 1h, wash the membrane with TBST 3 times, 5min each time; add diluted horseradish Peroxidase-labeled secondary antibody, react for 1 hour, wash the membrane 3 times, 5 min each time, and develop.

2.4 Statistical analyses

All data are expressed as mean-standard deviation and represent one of three independent experiments. Statistical analyses were performed using SPSS13.0. Treatment effects were analyzed using one-way analysis of variance followed by Dunnett's test. A value of $P < 0.05$ was used to indicate statistically significant differences.

3 Results

3.1 Component analysis

By searching SciFinder, PubMed, and CNKI databases, collecting and sorting documents related to compound data in Coptis, Phellodendron, Angelica, Rehmannia glutinosa, turmeric and sesame oil, using IDA (Information Association Data Acquisition) algorithm and MS/MSALL workflow of SWATHM acquisition software. Identify unknown compounds.

By comparing with the compound database, the candidate compound is obtained, and then the structure is determined by the possible fragmentation law of the secondary fragment ion and the precursor ion. Finally, by the comparison of the retention time of the commercially available reference substance, as well as the ultraviolet absorption spectrum and the high-resolution mass spectrum. The analysis of the data further confirms the results of some components. As a result, 43 components were identified, which are shown in Annex 1. Among them, mainly including berberine, palmatine, Coptisine, phellodendrine, ferulic acid, sesamol, sesamin, catalpol, curcumin, which are shown in Fig.1 and Fig.2.

3.2 Component analysis

Prepare 10 batches of test solution separately, take the test sample and the reference solution separately, record the chromatographic data, and import the chromatographic data into the "Chinese medicine chromatographic fingerprint similarity evaluation software (2012.130723 versions)". Reference with the S1 sample chromatogram, the median method was used to generate a control fingerprint (Fig.3), and 16 common peaks were determined. By comparing the retention times of the chromatograms of the mixed standard reference, eight peaks were identified, respectively. No.3 is phellodendron hydrochloride, No.4 is Sesamol, No.5 is ferulic acid, No.7 is coptisine, No.8 is berberine, No.9 is Palmatine chloride, No.15 is curcumin, and No.16 is Sesamin. The obtained compounds are the main active ingredients of CTCMDO. After consulting the literature, the above eight components all have different degrees of anti-inflammatory effects. [Tian, *et al*, 2021, Sang, *et al*, 2021]

3.3 Molecular docking

The MAPK signaling pathway is a key pathway for inflammatory diseases. It can transmit extracellular signals (such as stress, growth factors, etc.) into the cell, affecting tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6). cyclooxygenase 2 (COX-2) and other inflammatory factors participate in the regulation of cell proliferation, differentiation and apoptosis (Haruna, *et al*, 2021). MAPK directly or indirectly participates in TNF signaling pathway, NF-KB signaling pathway, MAPK signaling pathway and many other signaling pathways, forming complex interaction relationships (Francesco De Luca, 2021). Therefore, the targets of MAPK signaling pathway and NF-KB signaling pathway were selected for molecular docking verification. Download the target protein data file through the PDB database, and use the GOLD software to compare the fingerprints of the active ingredients of CTCMDO and the relevant target protein for molecular docking analysis. The results are shown in Fig.4.

3.4 Experimental validation

3.4.1 Effect of CTCMDO on Model of AD in Mice

Different concentrations of CTCMDO have significant differences in the treatment of mice. It is generally believed that the degree of ear swelling objectively reflects the degree of inflammation response, and the ear swelling of each group of mice is shown in Fig.5 Compared with the model group, the positive control group had a significant inhibitory effect on mouse ear swelling ($p < 0.01$), and the high-dose group had a

significant inhibitory effect on mouse ear swelling ($p < 0.01$), and the middle dose group could also be improved ear swelling in mice ($p < 0.05$).

The skin healing of each group of mice is shown in Fig.6. After all mice were induced by DNCB, the skin gradually thickened and crusted. With the treatment of CTCMDO and DEX, the molting of the back of the mice gradually fell off. It is getting smaller and smaller until it disappears. It can be seen from the figure that the molting of the 18th day positive group and the CTCMDO high dose group has completely fallen off. Body weights from all groups were recorded every 4 days, Combined with the actual situation and the results of each group's weight data, we found that the body weight of mice in the DEX group was significantly smaller than the other dose groups.

In order to evaluate the therapeutic potential of CTCMDO against AD, mice were treated with CTCMDO locally during the DNCB induction and sensitization phase. As shown in Figure 12, the DNCB group with fully developed skin damage showed hyperkeratosis and hyperplasia compared with the NOR group. DEX significantly inhibited the increase in thickness of the epidermis and dermis. In addition, CTCMDO treatment significantly inhibited the increase in epidermis, dermal thickness and inflammatory cell infiltration.(Fig.7)

3.4.2 Inhibitory effects of CTCMDO on substance P expressions

The positive staining of substance P is brown or brown, and it is mainly distributed in the cytoplasm or interstitium. (Alireza Mashaghi, et al.,2016) The results of immunohistochemistry are shown in Fig.8. The skin stimulated by DNCB showed effective staining of abundant substance P. On the contrary, compared with the DNCB group, repeated application of CTCMDO significantly inhibited the detection of substance P, and application of dexamethasone acetate also significantly inhibited the expression of substance P. The expression of substance P in the skin of the DNCB group was more, brown-yellow particles with darker coloration, and the positive expression was mainly located in the epidermis; the DEX group and the CTCMDO high and medium dose groups had only a small amount of expression, and the coloration was lighter. Compared with the DNCB group, the DEX group significantly inhibited substance P secretion, and the treatment of the high and medium dose groups of CTCMDO also significantly inhibited the expression of substance P. The results show that CTCMDO can reduce the allergic reaction during the onset of AD by inhibiting the expression of substance P.(J,J,Kang,et al,2021)

3.4.3Inhibition of effect of CTCMDO on cytokines

To evaluate the effect of CTCMDO on cytokine production, we measured INF- γ , TNF- α , IL-6, IL-4, IgE and IL-2 in serum. DNCB attack increases cytokine levels. Compared with DNCB-induced mice, the treatment of CTCMDO group and DEX group significantly improved the increase of INF- γ , TNF- α , IL-6, IL-4, IgE and IL-2 levels ($P < 0.05$ – $P < 0.01$; Fig.9).

3.4.4 The expression of MAPKp38 mRNA in the skin tissue of each group of mice

After one-way analysis of variance (Figure 10), the expression of p38MAPK mRNA in the skin tissues of the mice in each group was compared, and the difference

was statistically significant ($P \leq 0.05$); the pairwise comparison results showed that compared with the normal group, the skin of the model group was The expression of p-p38 MAPK mRNA in the tissue increased; compared with the model group, the expression of p-p38MAPK mRNA in the skin tissue of the CTCMDO treatment group decreased, and the difference was statistically significant ($P \leq 0.05$).

3.4.5 Inhibition effects of CTCMDO on MAPKS/NF- κ B expression

To further verify the mechanism of inhibiting inflammation, we evaluated the levels of MAPK (ERK, p38 and JNK) in the whole tissue lysate. Figure 14 shows that the use of DNCB caused a significant increase in MAPKs, while DEX treatment reduced the levels of p-ERK1/2, p-JNK and p-p38. Topical application of CTCMDO restored p-ERK1/2 and p-JNK, and p-p38 levels were reduced. Compared with the normal group, the expression of NF- κ B protein in the skin of the DNCB group was significantly increased ($P < 0.05$). Compared with the model group, the expression of NF- κ B65 protein in the skin tissue of the mice in the DEX group and the CTCMDO group the average level is decreasing. (Fig.11)

Discussion

DNCB is a commonly used method for the preparation of AD-like mice. The performance of this model mice is similar to that of clinical patients (Liu B. et al., 2016; Oetjen et al., 2017). This study used a mixture of 1% DNCB, acetone and olive oil to prepare the model. The results showed that the degree of ear swelling in the model group significantly increase; Compared with the model group, the ear swelling of the CTCMDO low, medium, and high dose groups and the dexamethasone acetate group was significantly reduced. Pathological sections indicated that the back skin of the mice in the model group was severely keratinized, edema, and a lot of eosinophilia Granulocyte infiltration is consistent with the preparation results of references AD-like models(Zhou, ZK et al., 2020), suggesting that the AD-like mice model was successfully prepared.

Substance P (SP) is a kind of eleven amino acid peptide that is ubiquitous in the human body. It is secreted by specific sensory nerve endings and responds to various stimuli(Mei, *et al.*,2020). This neuropeptide can regulate many pathophysiological processes, such as immune regulation and inflammation, Pain, itching, etc., play an important role in skin nerve activity, and are considered to be important inflammatory mediators that cause skin neurogenic pruritus. Studies have shown that substance P can mediate some inflammatory reactions and induce the body to produce itching, pain and other neural activities through its related G protein-coupled receptors. (Rubartelli, A,*et al.*,2013).Therefore, inhibiting the expression of substance P can help relieve the pain, itching and other symptoms that occur during the onset of eczema, and alleviate the suffering of patients. The results of immunohistochemical experiments showed that compared with the blank group, the expression of substance P in the skin lesions of mice in the eczema model group increased; compared with the model group, the expression of substance P in the skin lesions of the mice in the positive control group and the CTCMDO administration

groups Substance expression decreased ($P > 0.01$). The above results indicate that CTCMDO can reduce the occurrence of itching during the onset of eczema by inhibiting the expression of substance P.

In the animal experiment, CTCMDO exhibited protective effects against AD as shown by the histopathological results CTCMDO treatment significantly suppressed the increased thickness of the epidermis, dermis and the inflammatory cell infiltration (Fig. 13). More importantly, the effects of CTCMDO were comparable or better than the clinic drug dexamethasone acetate. In particular, we observed changes in the serum levels of various inflammatory cytokines involved in the pathogenesis of AD. TNF- α and IL-4 have been demonstrated as the primary cytokines initiating an inflammatory response, and their concentrations have been associated with AD severity (X. Wu. *et al.*, 2016). INF- γ is a multifunctional cytokine that plays an anti-inflammatory role in the inflammatory response (Chan *et al.*, 2013; Kabashima *et al.*, 2013). In the present study, the serum levels of the pro-inflammatory cytokines TNF- α , IL-4, IL-6, IgE, and INF- γ were elevated in the AD model mice, consistent with the results of previous studies (Kaesler, S *et al.*, 2014). However, the observed effects in the AD model mice were significantly suppressed by CTCMDO treatment. Therefore, the potential mechanism of CTCMDO in treating AD is probably by modulating the inflammatory responses in the infected tissues and cells. This postulation is partly consistent with our network pharmacology analysis results.

MAPK and NF- κ B signaling pathways are the two main approaches to regulate the release of inflammatory factors in skin lesion. Nuclear factor- κ B (NF- κ B) is a key transcription factor regulating Th2 cell differentiation and inflammation (Park *et al.*, 2017). The stimulation of internal/external factors activates the NF- κ B pathway, which leads to a significant increase in the level of inflammatory factors in the skin and serum, thereby aggravating the inflammatory process (Ayush *et al.*, 2013; Park *et al.*, 2017). Therefore, inhibiting NF- κ B activity is an important target for anti-inflammatory therapy. Western Blot experiment results show (Fig. 14), compared with the model group, topical application of CTCMDO inhibits the NF- κ B activity. MAPK signaling pathway is stimulated by intracellular inflammatory factors to stimulate phosphorylation, which leads to the production of inflammatory factors and promotes allergic inflammatory response, thereby activating the NF- κ B pathway (Ayush *et al.*, 2013; Park *et al.*, 2020). Therefore, inhibition of the MAPK pathway phosphorylation can reduce the synthesis of pro-inflammatory cytokines and intracellular signal transduction, down-regulate the activation of NF- κ B, thereby inhibiting the release of inflammatory factors and reducing the damage caused by inflammation to the skin lesions. Our results clearly show that CTCMDO exerts an anti-inflammatory effect by inhibiting MAPK phosphorylation and NF- κ B activation, and then blocking the production of multiple inflammatory factors, and alleviates a series of symptoms caused by inflammatory reactions.

Research shows that unlike some single medicines in Western medicine, most natural medicines or Chinese medicines contain multiple ingredients, which play a major role in therapeutic by acting on multiple targets (Bin, L *et al.*, 2016; Cho K *et al.*, 2018; Mohd.I.Yatoo, *et al.*, 2018). Therefore, this paper introduces modern techniques such as HPLC fingerprint, etc. To reveal the "spectrum-effect" relationship of CTCMDO and predict its mechanism of action. We analyzed the active ingredients of CTCMDO by HPLC and LC/MS. A total of 43 compounds were identified, including ferulic acid, berberine, berberine,

berberine, palmatine, catalpol, curcumin, sesamin, sesamol and other chemical components, and preliminary identification of the effective substances. The chromatogram of sample 1 (S1) was set as the reference map, and the fingerprint of the CTCMDO of 10 batches was established to exhibit 17 peaks. 8 chromatographic peaks were assigned, include: berberine, coptisine, palmatine, Phellodendrine, ferulic acid, curcumin, sesamol and sesamin. Modern research shows that the above ingredients have good therapeutic effects on the treatment of AD (Bin, L *et al.*, 2016; Cho K *et al.*, 2018;Xiao et al.,2018), which is in line with the advantages of the “multi-component, multiple targets,multi-pathways” of traditional Chinese medicine.

Conclusion

This study combines the existing literature and research progress, application of modern experiments to research on the efficacy and mechanism of CTCMDO in the treatment of AD. The results showed that CTCMDO can improve AD by inhibiting the activation of two signaling pathways (MAPK and NF- κ B), andinhibiting the production of inflammatory factors. Compared with dexamethasone acetate, CTCMDO has the advantages of multi-ingredients, multi-targets and multi-pathways treatment. CTCMDO has a unique prevention and treatment effect, no obvious adverse reactions and no drug tolerance can develop after long-term use. It is a valuable asset of Chinese medicine and is worthy of our exploration and development. Taken together, our results might provide an important scientific basis for the development of therapeutic agents against AD and could provide a more appropriate starting point for the future search for preventative measures and treatments for AD.

Abbreviations

HLG: huang-lian-ointment; DNCB: 1-chloro-2,4-dinitrobenzen; TCM: Traditional Chinese medicine; CAM: complementary and alternative medicine; HPLC:high performance liquid chromatography; AD: atopic dermatitis; IgE: Immunoglobulin E; TNF- α : tumor necrosis factor- α ; INF- γ : Interferon- γ ; MAPKs: mitogen-activated protein kinases; NF-KB: nuclear factor KB;IL-6: interleukin-6, IL-2: Interleukin-2; IL-4: Interleukin-4.

Declarations

Disclosure of conflicts of interest

We declare that there are no conflicts of interest associated with this manuscript and no significant financial support that would influence our findings.

Author contributions

Z.R.R., Y.M.M. and Z.D.Q. conceived and designed experiments. Z.R.R., S.Y.X. and L.G.Z. performed experiments.Z.R.R. and S.S. interpreted the data and wrote the original manuscript. S.J.M., Y.M.M. and Z.D.Q. provided helpful discussions and refined the paper.

Ethics approval and consent to participate

The animal protocol was approved by the Animal Care Ethics Committee of Changchun University of Chinese Medicine (Changchun, Jilin, China, Approval No. 20181011).

Consent for publication

The manuscript is approved by all authors for publication.

Data Availability Statement

The data sets supporting the conclusions of this article are included within the article and its additional files.

Funding

This study was supported by the grants from the National Key Research and Development Program: research on key technologies of ginseng industry and development of massive health products (2017YFC1702100); The Science and Technology Development Plan Project of Jilin Province (20180623041TC); Jilin Province Education Department Project (JJKH20210970KJ).

Competing interests

We declare that there are no conflicts of interest associated with this manuscript and no significant financial support that would influence our findings.

References

1. Ayush, O., Lee, C. H., Kim, H. K., Im, S. Y., Cho, B. H., and Lee, H. K. (2013). Glutamine suppresses DNFB-induced contact dermatitis by deactivating p38 mitogen-activated protein kinase via induction of MAPK phosphatase-1. *J. Invest. Dermatol.* 133, 723–731.
2. Alireza Mashaghi¹, Anna Marmalidou¹, Mohsen Tehrani¹, Peter M. Grace², Charalabos Pothoulakis³, Reza Dana. (2016) *Cellular and Molecular Life Sciences*. (2016) 73:4249–4264
3. Bin, L., Leung, D.Y., 2016. Genetic and epigenetic studies of atopic dermatitis. *Allergy Asthma Clin. Immunol.* 12, 52.
4. Cao, H., Li, S., Xie, R., Xu, N., Qian, Y., Chen, H., Hu, Q., Quan, Y., Yu, Z., Liu, J., 2018. Exploring the mechanism of Dang gui liu huang decoction against hepatic fibrosis by network pharmacology and experimental validation. *Front. Pharmacol.* 9, 187.
5. Chan, C.C., Liou, C.J., Xu, P.Y., Shen, J.J., Kuo, M.L., Len, W.B., Chang, L.E., Huang, W.C., 2013. Effect of dehydroepiandrosterone on atopic dermatitis-like skin lesions induced by 1-chloro-2,4-dinitrobenzene in mouse. *Journal of Dermatological Science* 72, 149-157.

6. Cho K, Parveen A, Kang MC, Subedi L, Lee JH, Park SY, Jin MR, Yoon H, Son YK, Kim SY. *Pyrusussuriensis* Maxim.leaves extract ameliorates DNCB-induced atopic dermatitis-like symptoms in NC/Nga mice., *Phytomedicine : international journal of phytotherapy and phytopharmacology*2018 Sep; 48:76-83.
7. De Benedetto, A., Kubo, A., Beck, L.A., 2012. Skin Barrier Disruption: A Requirement for Allergen Sensitization *The Journal of Investigative Dermatology* 132, 949-963.
8. David Boothe W, Tarbox JA, Tarbox MB.(2017). Atopic Dermatitis: Pathophysiology.*Adv Exp Med Biol.* 2017;1027:21-37.
9. Francesco De Luca.(2021).Regulatory role of NF- κ B in growth plate chondrogenesis and its functional interaction with Growth Hormone.*Molecular and Cellular Endocrinology.*514(2020)110916.
10. Guo, Q., Zheng, K., Fan, D., Zhao, Y., Li, L., Bian, Y., Qiu, X., Liu, X., Zhang, G., Ma, C.,2017. Wu-Tou decoction in rheumatoid arthritis: integrating network pharmacology and in vivo pharmacological evaluation. *Front. Pharmacol.* 8, 230
11. Haruna Nagao , Daisuke Kitagawa , Fumio Nakajima, Masaaki Sawa , Takayoshi Kinoshita, (2021).Identification of an allosteric and Smad3-selective inhibitor of p38 α MAPK using a substrate-based approach.*Bioorganic & Medicinal Chemistry Letters.*43 (2021) 128056
12. Jinjoo Kang, Soyoung Lee , Namkyung Kim , Hima Dhakal, Young-Ae Choi , Taeg Kyu Kwon, Dongwoo Khang, Sang-Hyun Kim.(2021)Hispidulin alleviates 2,4-dinitrochlorobenzene and house dust mite extract-induced atopic dermatitis-like skin inflammation.*Biomedicine & Pharmacotherapy.*137 (2021) 111359
13. Kaesler, S., Volz, T., Skabytska, Y., Koberle, M., Hein, U., Chen, K. M., et al. (2014). Toll-like receptor 2 ligands promote chronic atopic dermatitis through IL-4-mediated suppression of IL-10. *J. Allergy Clin. Immunol.* 134, 92–99.
14. Leung, D.Y.M., 2013. New Insights into Atopic Dermatitis: Role of Skin Barrier and
15. Immune Dysregulation. *Allergology International* 62, 151-161.
16. Meilian Yang, Yudan Wang, et al. (2020).In vitro and in vivo anti-inflammatory effects of different extracts from *Epigynum auritum* through down-regulation of NF- κ B and MAPK signaling pathways .*Journal of Ethnopharmacology*,2020.113105
17. Mohd. I.Yatoo¹, Arumugam Gopalakrishnan, Archana Saxena,Oveas R. Parray, Noore A. Tufani, Sandip Chakraborty, Ruchi Tiwari, Kuldeep Dhama Hafiz M.N. Iqba.(2018)Anti-Inflammatory Drugs and Herbs with Special Emphasis on Herbal Medicines for Countering Inflammatory Diseases and Disorders - A Review.*Recent Pat Inflamm Allergy Drug Discov.* 2018;12(1):39-58
18. Oetjen, L. K., Mack, M. R., Feng, J., Whelan, T. M., Niu, H., Guo, C. J., et al. (2017). Sensory neurons co-opt classical immune signaling pathways to mediate chronic itch. *Cell* 171, 217–228.e13.
19. Park, J.U., Kang, B.Y., Lee, H.J., Kim, S., Bae, D., Park, J.H., et al. 2017. Tetradecanol reduces IL-4 T cell growth by the down regulation of NF-kappaB mediated IL-2secretion. *Eur.J. Pharmacol.* 799,135–142.

20. Park, JH , Hwang, MH , Cho, YR, Hong, SS , Kang, JS , Kim, WH , Yang, SH , Seo, DW (Seo, Dong-Wan), Ahn, EK., 2020. Combretum quadrangulare Extract Attenuates Atopic Dermatitis-Like Skin Lesions through Modulation of MAPK Signaling in BALB/c Mice. MOLECULES.25(8).
21. QN,Sang, QQ,Jia, HY, Zhang, CH, Lin, XD,Zhao, M,Zhang, YR, Wang, P, Hu,(2021)Chemical profiling and quality evaluation of Zhishi-Xiebai-Guizhi
22. Decoction by UPLC-Q-TOF-MS and UPLC fingerprint.Journal of Pharmaceutical and Biomedical Analysis.194 (2021) 113771.
23. Rubartelli, Anna ,Lotze, Michael T. et al.(2013)Mechanisms of sterile inflammation.Frontiers in Immunology.4(2013)398.
24. Torres T, Ferreira EO, Gonalo M, Mendes-Bastos P, Selores M, Filipe P.(2019)Update on Atopic Dermatitis.Acta Med Port. 2019 Sep 2;32(9):606-613
25. T, Tian, X,Xu, X, Li, WH, Zhang , HT, Lu.(2021)Precision-characterization and quantitative determination of main compounds in Si-Ni-San with UHPLC-MS/MS based targeted-profiling method.Journal of Pharmaceutical and Biomedical Analysis.194 (2021) 113816.
26. Weidinger, S. & Novak, N. Atopic dermatitis. Lancet 387, 1109-1122 (2016).
27. Liu, B., Tai, Y., Achanta, S., Kaelberer, M. M., Caceres, A. I., Shao, X., et al. (2016). IL-33/ST2 signaling excites sensory neurons and mediates itch response in a mouse model of poison ivy contact allergy. Proc. Natl. Acad. Sci. U.S.A. 113, E7572–E7579.
28. Xiao, Z., and Xiao, S. (2018). The anti-inflammatory effect of fructus kochiae on allergic contact dermatitis rats via pERK1/2/TLR4/NF-kappaB pathway activation. Evid. Based Complement. Alternat. Med. 2018:1096920.
29. X,Wu.X,Feng,Y.He,Y.Gao,S.Yang,Z.Shao,C.Yang,H.Wang,Z,Ye .(2016).IL-4 administration exerts preventive effects via suppression of underlying inflammation and TNF-alpha-induced apoptosis in steroid-induced osteonecrosis.Osteoporos Int. 2016 May;27(5):1827-37.
30. YJ, Qi , H,Zhang, DM,Wang , GT, Ding , ZR, Ma, XY, Zhou, XP, Luo, NZ,Jin, MY, Wang, HN, Lu., (2021).Antibacterial efficacy and molecular docking analysis of Huang-Lian-Jie-Du Decoction against the phytopathogenic bacteria P. carotovorum PC1.Journal of Molecular Structure.1234 (2021) 130141
31. Yu, G., Wang, W., Wang, X., Xu, M., Zhang, L., Ding, L., Guo, R., Shi, Y., 2018. Network pharmacology-based strategy to investigate pharmacological mechanisms of Zuo jin wan for treatment of gastritis. BMC. Med. 18 (1), 292.
32. Zhou, ZK (Zhou, Zhike), Shi, TX, Hou, J, Li, M (Li, Min)., 2020. Ferulic acid alleviates atopic dermatitis-like symptoms in mice via its potent anti-inflammatory effect. Immunopharmacology and immunotoxicology. DOI:10.1080/08923973. 2020. 1733012.

Figures

Figure 1

Targeted-MS/MS spectra of 4 identified compounds: (A) Ferulic acid, (B) Berberine, (C) Curcumin, (D) Sesamin.

Figure 2

Targeted-MS/MS spectra of 4 identified compounds: (A) Palmatine, (B) Phellodendrine, (C) Sesamol, (D)Coptisine, (E)Sesamin.

Figure 3

The high performance liquid chromatography (HPLC) profile and quality control analysis of CTCMDO using UV detection at 278 nm

Figure 4

Docking analysis of four important targets of CTCMDO in the treatment of AD: A-ERK; B-JNK; C-NF-kB P65; D-MAPK P38

Figure 5

Effect of CTCMDO on ear swelling in mice (Mean±SD) **P<0.01, *P<0.05 compared with the DNCB group

Vehicle:DNCB sensitization

Positive group:administration with DEX

High group:administration with HLG(7.5g/kg)

Med group:administration with HLG(5.0g/kg)

Low group:administration with HLG(2.5g/kg)

Figure 6

Wound healing performance of mice in different groups; Body weights from all groups were recorded every 4 days and shown as mean $\pm$ S.D.

Figure 7

Pathological sections of mice in each group (HE×200)

NOR

DNCB

DEX

HIGH

MED

LOW

Figure 8

Effects of AS on DNCB-induced scratching behavior and substance P expression.

Figure 9

Effects of CTCMDO on DNCB-induced cytokine levels: interleukin INF-γ, TNF-α, IL-6, IL-4, IgE and IL-2 in mice. Values of cytokines are mean–standard error of the mean. ##P<0.01 compared with the NOR group; **P<0.01 and *P<0.05 compared with the DNCB group.

Figure 10

Comparison of p38MAPK expression in skin tissue of mice in each group—Compared to MOD group—* $P < 0.05$

Figure 11

Effects of CTCMDO on DNCB-induced expressions of pro-inflammatory markers in mice. ERK1/2, p38, JNK and NF-κB65 level. The pro-inflammatory expression was determined by western blot analysis. The results are representative of three separate experiments. ##P<0.01 compared with the NOR group; **P<0.01, *P<0.05 compared with the DNCB group.