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Original Research Article

Antiallergic effects of ethanol extract of *Cnidium monnieri* (L.) Cuss. on DNCB-induced atopic dermatitis in mice

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Abstract

Purpose: *To study the anti-allergic effects of ethanol extract of Cnidium monnieri (L.) Cuss. on 2, 4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis in mice.*
Method: *Atopic dermatitis (AD) was induced by DNCB in Balb/c mice, and the mice randomly divided into normal group, negative control group, hydrocortisone group, and ethanol extract of Cnidium monnieri (L.) Cuss. (EECM) group. Ear swelling was determined by measuring the thicknesses of the left and right ears of each mouse. Spleen and thymus indices were calculated from spleen, thymus and body weight values. The levels of TNF-α and IgE in serum were determined by enzyme-linked immunosorbent assay (ELISA). Hematoxylin-eosin (H & E) staining and toluidine blue staining were used to evaluate pathological changes in ear tissue, while high performance liquid chromatography (HPLC) was performed to ascertain the bioactive compounds in EECM.*
Results: *Compared with the negative control group, EECM significantly alleviated skin lesions, reduced thickness of ear swelling, and decreased spleen and thymus indexes of mice ($p < 0.05$). Moreover, EECM significantly reduced epidermal thickness ($p < 0.01$). However, EECM did not significantly alter the number of mast cells ($p > 0.05$). The expressions of TNF-α and IgE in serum were also significantly down-regulated ($p < 0.01$, $p < 0.05$). Results from HPLC revealed that the contents of bergapten, imperatorin and osthole in EECM were 0.73, 3.69 and 9.40 mg/g, respectively.*
Conclusion: *EECM ameliorates AD in mice via inhibition of inflammation and by a mechanism that might be related to the regulation of TNF-α and IgE levels. The major bioactive constituents of EECM are osthole, imperatorin and bergapten. Thus, this plant extract has a potential to be developed for the treatment of of atopic dermatitis.*

Keywords: *Cnidium monnieri, Atopic dermatitis, Anti-allergy, HPLC, Osthole, Imperatorin, Ergapten*

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INTRODUCTION

AD is an inflammatory skin disease which manifests clinically as dry skin, severe pruritus and eczema-like lesions and impaired skin barrier function [1]. Epidemiological investigations have revealed that the incidence of AD is up to 20 and 8 % in infants and adults, respectively [2]. The disease seriously affects the quality of life of the affected patients. It has been found that genetic variation, environmental stimulation and their interaction may lead to

disorders in the skin microenvironment, thereby enhancing the development of AD. Results from a study indicates that the genetic sensitivity, induction of environmental factors, Th2 cell-dominated immune response and skin barrier dysfunction may be involved in the pathogenesis of AD [3]. Currently, the first-line therapy drugs for AD are mostly topical glucocorticoids and calcineurin inhibitors. However, the long-term use of these drugs is restricted due to their associated side effects such as skin atrophy, hirsutism, burning sensation, and irritation. Therefore, there is need for development of newer AD drugs with fewer adverse reactions and better efficacy than the current ones in use.

To the best of our knowledge, traditional Chinese medicine (TCM) is characterized by its unique effects. Moreover, results from modern pharmacological investigations indicate that TCM has potent biological properties such as immunoregulatory, anti-tumor and antioxidant effects [4]. In China, the fructus of *Cnidium monnieri* (L.) Cuss. has been frequently used to treat eczema, pruritus and other skin diseases due to its efficacy in *dispelling wind*, *drying dampness* and *killing parasites* to relieve itch. Moreover, lots of Chinese herbal prescriptions for treating AD contain *Cnidium monnieri* (L.) Cuss. However, only very few studies have focused on the pharmacological effect of the extract of *Cnidium monnieri* (L.) Cuss. on AD [5]. Based on aforementioned, it was hypothesized that the EECM might ameliorate DNCB-induced AD. Therefore, the present research was to study the beneficial effects of EECM on AD mice, and to identify the bioactive compounds in EECM that might be responsible for any observed effects.

EXPERIMENTAL

Materials and reagents

Cnidium monnieri (L.) Cuss. was obtained from Xinhehua Company at Sichuan Province of China, and it was identified by a taxonomist, Professor Chunjie Wu at Chengdu University of TCM. Standards for osthole, imperatorin and bergapten were provided by the Chengdu Must Bio-Technology Co. Ltd (Sichuan province, China). Assay kits for TNF-α and IgE ELISA were acquired from the ExCell Bio (Shanghai, China), while 2, 4-dinitrochlorobenzene (DNCB) was gained from the Sigma (St, Louis, Mo, USA).

Animals

Male SPF Balb/c mice (18 - 22 g) were obtained from the Chengdu Dossy Experimental Animals Co. Ltd. (SCXK: 2014-124, Chengdu, China). The mice were fed in the laboratory at temperature range of 22 - 25 °C and humidity range of 50 – 60 %. The animals were permitted *ad libitum* access to feed and water. Meanwhile, all the process of experiment was conducted following ethical approval from the Committee for Animal Care and Use of Laboratory Animals, College of Pharmacy, Chengdu University of Traditional Chinese Medicine and, and the study followed NIH Guidelines for Care and Use of Laboratory Animals (NIH publication no. 85 - 23, revised 1985) [6].

Preparation of ethanol extract of *Cnidium monnieri* (EECM)

Based on a previous report [7], 75 % ethanol (500 mL) was used to extract the coumarins derived from the powder of *C. monnieri* (100 g). Then, the filtrate was dried in a rotary evaporator, and the dry extract was dissolved in ethylene glycol to achieve a stock concentration of 437 mg/mL.

HPLC analysis

The coumarin contents of EECM were determined using HPLC (Shimadzu LC-10A, Japan) with chromatographic column (Eclipse Plus-C18, 250mm×4.6mm, 5μm). The chromatographic conditions were as follows: mobile phase of acetonitrile A-water B, gradient elution (0-30 min, 35% A, 65% B; 31～60min, 55% A, 45% B); flow rate of 0.8 mL/min; detection wavelength of 320 nm and column temperature of 35 °C [8]. The EECM was dissolved in methanol to acquire a concentration of 0.9352 mg/mL which was injected at an injection volume of 10 μL. Three independent experiments were performed to analyze the bioactive contents of EECM.

Animal studies

Balb/c mice were randomly divided into normal group, negative control group (untreated AD), hydrocortisone group and EECM group, with 10 mice in each group. The procedure used for establishing DNCB-induced AD model was based on a method established in a previous study [9]. An abdominal area of each mouse (approximately 2×2 cm^2) was shaved for sensitization of the mouse skin with 7 % DNCB in acetone/olive oil (v/v, 3:1). Then the 0.5 % DNCB (20 μL) was topically used to induce the AD-like skin lesion by repeated application on the right ear of mouse. After the AD model was successfully established, 0.1 % hydrocortisone (0.1 g/mouse) and EECM (13.1 mg/mouse) was topically used to the right ear of each mouse in

hydrocortisone group and EECM group, respectively, once daily for 7 days.

Measurement of thickness of ear swelling

The thicknesses of the left and right ears of mice were measured using a random single-blind method. The thickness of ear swelling (T) was calculated as shown in Eq 1.

T (mm) = Tr – Tl) ……. (1)

where Tr and Tl are the thickness of right and left ear of the mice, respectively.

Organ index

Fresh spleen and thymus were weighed, and the values obtained were used to calculate each organ index (G), as in Eq 2.

G = (W/Wb) …… (2)

where W and Wb are organ and body weights, respectively.

Histopathologic examination

A portion of the right ear of each mouse was fixed in the 4 % paraformaldehyde for 24 h. Then, the tissues was processed for light microscopy and stained with H & E, followed by staining with toluidine blue. Image-pro plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the epidermal thickness in five consecutive fields of the epidermis, and for calculating the amount of mast cells in three continuous fields under a light microscope at x200 magnification. The number of mast cells per square millimeter was calculated in each of the fields under the microscope.

ELISA

The levels of TNF-α and IgE were determined in mouse serum using ELISA kits according to the instructions of the kit manufacturers.

Statistical analysis

The SPSS19.0 software was used for data processing. Statistical analysis was done using One-way analysis of variance (ANOVA). The LSD and Dunnett's *t*-tests were used for multiple group comparisons. $P < 0.05$ was regarded as indicative of statistically significant difference.

RESULTS

Coumarin contents of EECM

Results from HPLC showed that the main bioactive compounds in EECM were coumarins. The contents of bergapten, imperatorin and osthole in EECM were presented in Figure 1. The regression equations and linearity ranges for bergapten, imperatorin and osthole are shown in Table 1. As showed in Table 2, and the levels of bergapten, imperatorin and osthole were 0.73, 3.69 and 9.40 mg/g, respectively.

Effects of EECM on thickness of ear swelling in mice with AD

As shown in Figure 2, red swelling, hypertrophy and scabbies in the right ear of control mice were obviously observed on day 11, which indicated that the AD skin lesions induced by DNCB were successfully established. On day 17, compared to the normal group, the thickness of ear swelling in negative control group was significantly increased ($p < 0.01$). However, compared with the control group, the topical treatment with EECM and hydrocortisone markedly relieved the skin lesions and ear swelling ($p < 0.01$).

Table 1: Regression equations and linearity ranges of bergapten, imperatorin and osthole

Compound	Regression equation	R^2	Linearity range (mg/mL)
Bergapten	$Y=7.206\times10^4+4.513\times10^7X$	0.9989	0.0025-0.0400
Imperatorin	$Y=2.167\times10^5+2.173\times10^7X$	0.9981	0.0125-0.2004
Osthole	$Y=1.127\times10^6+3.827\times10^7X$	0.9973	0.0312-0.5000

Table 2: Levels of bergapten, imperatorin and osthole in EECM in 3 independent assessments (mg/g)

Sample extract	Bergapten	Imperatorin	Osthole
1	0.74	3.7	9.52
2	0.72	3.66	9.34
3	0.72	3.66	9.34
Mean	0.73 mg/g	3.69 mg/g	9.40 mg/g

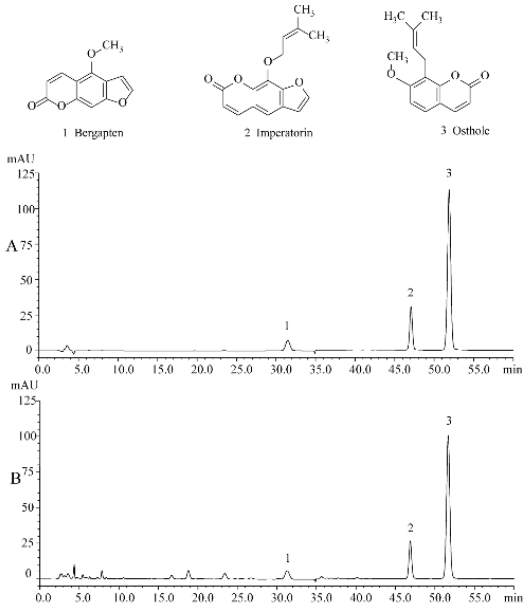

Figure 1: HPLC chromatogram of EECM (A: Standard B: Sample)

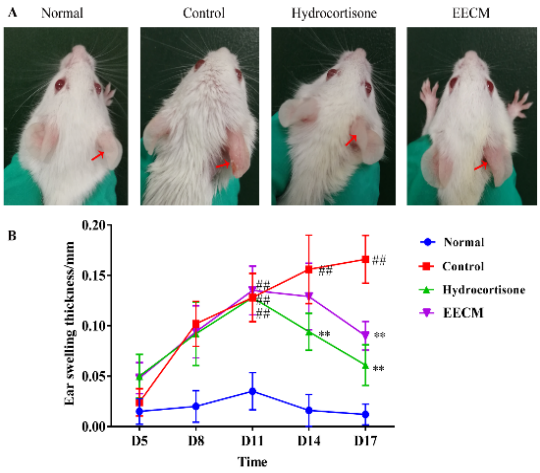

Figure 2: Effect of EECM on ear swelling thickness in mice with AD. Values are expressed in mm, and as mean ± SD (*n*=10)

Effect of EECM on spleen and thymus indices in mice with AD

To study the effects of EECM on the immune response of AD, the spleen and thymus index were evaluated, as presented in Figure 3. Compared to the normal group, the spleen and thymus index were markedly elevated in control group mice ($p < 0.05$, $P<0.05$). However, compared to the control group, the administration of hydrocortisone and EECM led to significant reductions in these indexes ($p < 0.05$).

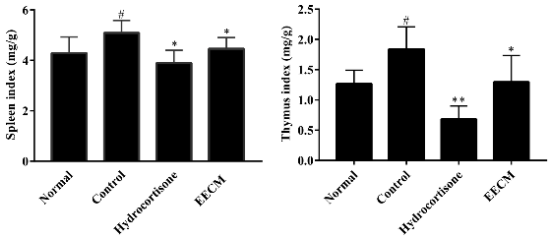

Figure 3: Effect of EECM on organ index in mice with AD. Values are presented as mg/g, and as mean ± SD, *n*=10)

Effect of EECM on ear histopathology of mice with AD

The results of histopathology of right ear of mice were shown in Figure 4. Compared with the normal group, the epidermal thickness (red mark) and number of mast cells (black arrow) were significantly increased in negative control group mice ($p < 0.01$). In contrast, these deleterious changes were markedly reversed after treatment of the mice with hydrocortisone ($p < 0.05$). However, EECM treatment decreased epidermal thickness, but had no effect on the number of mast cells.

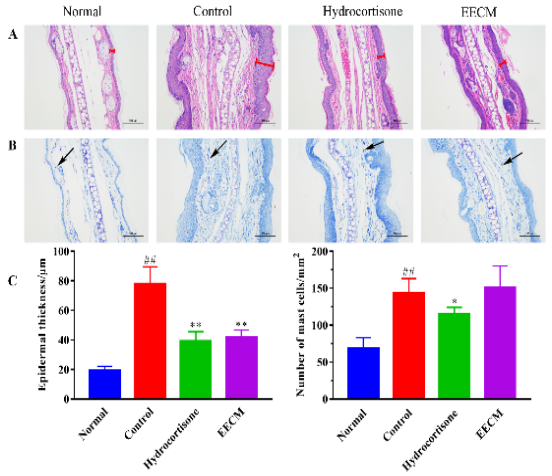

Figure 4: Effects of EECM on histopathology of ear tissue in AD mice. Values are expressed as mean± SD, n =10; x200; scale = 100 μm)

Effects of EECM on expressions of TNF-α and IgE in serum of mice with AD

The expression of TNF-α and IgE were determined by ELISA assay. As is shown in Figure 5, the levels of TNF-α and IgE were significantly up-regulated in negative control group, when compared to the normal group. However, the topical hydrocortisone and EECM treatments markedly reduced the TNF-α and IgE

levels. Thus, the EECM exerted the anti-allergic effects on DNCB-induced AD in mice.

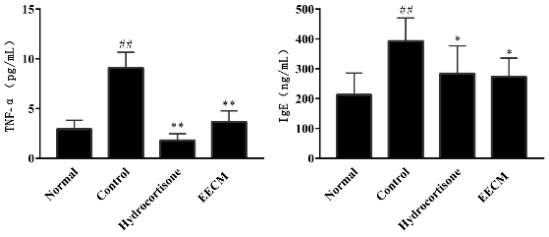

Figure 5: Effect of EECM on serum levels of TNF-α and IgE in AD mice. Values are presented as mean ± SD (n = 6)

DISCUSSION

Previous studies had demonstrated that the pathogenesis of AD was connected with immune imbalance mediated by Th1/Th2/Th22/Th17, skin barrier dysfunction induced by decreased filaggrin and disorder of skin flora caused by the dominant *Staphylococcus* aureus [10,11]. Although great advances on AD has been made, the eczema-like lesions and repeated pruritus still seriously affect the lives of AD patients. For example, the emerging Dupilumab (IL-4/IL-13 receptor antibody) and Baricitinib (JAK inhibitor) produced good outcomes in a clinical study [12]. However, the first-line treatment drugs for AD i.e., glucocorticoids, are associated with obvious adverse reactions which make them unsuitable for long-time use. Thus, there are lots of limitations and difficulties in the current treatment of AD. These impediments need to be addressed through further studies.

Traditional Chinese medicine has for long been used for used for topical treatment of dermatitis and eczema, with very good efficacy and very limited adverse reactions. Previous investigations suggested that the Shenshe Lotion and Shehuang Ointment exerted significant protective effects against AD-induced skin lesions, inflammation and pruritis in mice [9]. The Huang-Lian-Jie-Du Decoction, a well-known traditional herbal formula, was found to alleviate AD-mediated skin lesions in mice through down-regulation of IL-4 and TNF-α via suppression of the MAPKs/NF-κB pathway [13]. In addition, the results of Meta-analysis implied that the *Cnidium monnieri* (L.) Cuss. was a high frequency Chinese herbal medicine for topical treatment of AD in China [14]. Further, the latest study had revealed that the ethyl acetate extract from *Cnidium monnieri* (L.) Cuss. exhibited marked antipruritic effect on mice with 2,4-dinitrofluorobenzene (DNFB)-induced AD [15]. Thus, this study was to investigate the protective effect of EECM against DNCB-induced AD mice, and to elucidate the potential mechanisms involved.

The results indicated that EECM improved the skin lesions, suppressed the excessive immune response and anti-allergy activity. As described in Figure 2, the management of EECM was able to clearly weaken the swelling and scab of right ear. Moreover, the results of histopathology also manifested that the epidermal thickness and hyperkeratosis were markedly inhibited by EECM. In the AD model mouse, the stratum spinosum showed evident thickening, which is usually regarded as the classical pathological change in AD. The abnormal epidermal layer resulted in impairment of skin barrier function which aggravated itching and stimulation of allergens. However, there was no obvious change in the number of mast cells, when compared to the negative control group. This suggests that the beneficial effects of EECM against AD might be mediated through other immune cells rather than mast cells.

A recent study revealed that basophils-mediated leukotriene C4-CysLTR2 neuronal signaling was responsible for the acute allergen-induced itch in AD [16]. The spleen and thymus index also were notably restrained after the treatment of EECM, which was related to the immunosuppressive activity of EECM. Furthermore, ELISA results suggested that the elevated TNF-α and IgE levels were markedly reversed by EECM. Hence, EECM exerted significant anti-inflammatory and anti-allergic pharmacological effects on DNCB-induced AD in mice. In another study, it was also demonstrated that coumarins derived from *Cnidium monnieri* (L.) Cuss. exhibited widespread pharmacological activities, e.g., anti-inflammation, anti-allergy, anti-asthma [17]. It is known that the major bioactive compounds in *Cnidium monnieri* (L.) Cuss. are coumarins. using HPLC. Therefore, in this study, the contents of bergapten, imperatorin and osthole in EECM were determined by HPLC. As shown in Table 2, the main compounds in EECM were osthole and imperatorin. A study has shown that osthole produced anti-allergic and immuno-modulatory effects on ovalbumin-induced asthma in mice via inhibition of the production of Th1/Th2 cytokines, and promotion of the maturation of dendritic cells derived from bone marrow [18]. Based on the results of this study, it can be reasonably inferred that EECM exerted anti-inflammatory and anti-allergic effects on DNCB-induced AD in mice, most likely due to the presence of the bioactive compound osthole. However, the specific mechanisms and signaling

pathways involved in this process need to be further clarified in subsequent investigations.

CONCLUSION

The findings of this study indicate that EECM exerts significant anti-inflammatory, anti-allergic effects on AD mice. The specific mechanisms of action might be related to the reduction of TNF-α and IgE, which may be attributed to the active components of the extract (osthole, imperatorin and bergapten). However, there is need for further studies to elucidate the specific signaling pathways and potential key targets involved in the EECM-mediated mitigation of AD.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaoyuan Zheng conceived and designed the study. Yao He and Jiaoni Zheng collected the data while Meiling Xu and Chonghui Dan analysed the data. Zhijie Yu wrote the manuscript. All authors read and approved the manuscript for publication.

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