



Study on the Treatment of Experimental Dermatitis with *Ginkgo biloba* Leaf Extract and Quercetin

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ABSTRACT

Allergic contact dermatitis (ACD), is a classic delayed allergic reaction mediated by cellular immunity. P815 degranulation model was established by stimulating P815 cells with C48/80 inducer to explore the effect and mechanism of quercetin on mast cell degranulation. The ACD model of mice was established by 1-Chloro-2,4-dinitrobenzene (DNCB) external application to explore the therapeutic effect and mechanism of quercetin and *Ginkgo biloba* leaves extract on ACD. Quercetin could inhibit degranulation of P815 mast cells and significantly reduced the concentration of β -Hex, Histamine (HIS), IL-4 and TNF- α in the supernatant of degranulated cells ($P < 0.05$), significantly decreased IL-4, TNF- α , Pik3r3, Akt2, Gsk-3 β mRNA expression ($P < 0.05$). *G. biloba* leaf extract and quercetin could significantly reduce the levels of IL-6, IL-4, IL-1 β , TNF- α and IgE ($P < 0.05$), the expression levels of TLR4, NF- κ B1 and TNF- α were significantly decreased ($P < 0.05$). Quercetin and *G. biloba* extract have good therapeutic effect on DNCB induced ACD mice.

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Authors' Contribution

MX performed experiments. MH designed the study. JD analyzed the data. DW wrote and revised the manuscript.

Key words

Ginkgo biloba leaf extract, Quercetin, ACD, TLR4, P815 mast cells

INTRODUCTION

Allergic contact dermatitis (ACD), an acute or chronic inflammatory reaction of the skin caused by contact of the skin or mucous membrane with some external sensitizing substances (dust mites, pollen, parasites, food, etc.), is characterized by damage, swelling, edema, pruritus and ulceration of the skin or mucous membrane (Henderson *et al.*, 2001). A variety of cytokines (IL-6, IL-1 β , TNF- α , etc.), chemokines (CXC, CC, CX3C, etc.) and costimulatory molecules participate in the inflammatory process. Hormone drugs (dexamethasone, methylprednisone, etc.) and antihistamines (kairuitan, chlorpheniramine, etc.) are commonly used in the clinical treatment of ACD (Doucet *et al.*, 2002). However, several studies have reported that patients have a high recurrence rate, strong side effects, and drug resistance after long-term treatment with western medicine (O'Hara *et al.*, 2010).

In addition, hormone drugs are harmful to the liver, kidney and other important organs. In contrast, Chinese medicine has the advantages of widely available sources, low costs, and fewer adverse reactions.

Ginkgo biloba, a deciduous tree, belongs to the Ginkgo family. Its roots, pollen, seed coat, kernel and leaves have pharmacological activities, such as antihyperlipidemia (Adisakwattana *et al.*, 2012), anti-inflammatory, antiviral (Lee *et al.*, 2015) and antiallergy activities (Wu *et al.*, 2011). Quercetin (C₁₅H₁₀O₇) is a flavonoid compound with high concentrations in *G. biloba* extract, which has biological activities such as antioxidant, anticancer, and anti-inflammatory activities (Wong and Chiu, 2011). Studies have proven that quercetin can significantly increase the activities of serum T-AOC (total antioxidant capacity), GSH-Px (glutathione peroxidase) and SOD (superoxide dismutase) in the D-galactose-induced aging mouse model and reduce the malondialdehyde (MDA) content (Molina *et al.*, 2003). In addition, quercetin plays an important role in inhibiting the growth of many cancer cells (colon cancer, liver cancer, breast cancer, lung cancer, etc.) (Kim *et al.*, 2005). In the LPS-induced RAW264.7 inflammatory cell model, quercetin significantly reduced the release of reactive oxygen species (ROS) and monocyte chemotactic protein-1 (MCP-1), and selectively inhibited cyclooxygenase-2 (COX-2) (Guo *et al.*, 2012). Previous studies have found that the tumor necrosis factor - α (TNF- α) and interleukin 6 (IL-6) levels decreased in rats

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with autoimmune uveitis after quercetin administration, and the mechanism was related to the Wnt/ β -catenin signaling pathway (Arumugam *et al.*, 2012).

Mast cells are immune cells involved in allergic reactions that can degranulate and release chemokines, lipid mediators and cytokines under the activation of compounds 48/80 (Chatterjea *et al.*, 2012). The release of histamine (HIS) leads to an increase in vascular permeability and contraction of smooth muscle (Mostowy and Cossart, 2012). Similarly, the release of cytokines (IL-4, IL-13, etc.) can stimulate and amplify the Th2-type response. TNF- α can promote inflammation and further stimulate a variety of cells to produce cytokines (Howell *et al.*, 2008). The DACD mouse model, induced by dinitrobenzene (DNCB), is a classic model used to evaluate the anti-allergic effect of drugs (Lim *et al.*, 2015).

The chemical components of *G. biloba* extract are complex and mainly include flavonoids and lactones; hence, there are many pharmacological targets. In this study, we selected the representative active component of *G. biloba* extract and evaluated the regulatory effect of quercetin on allergic reactions *in vitro*. We compared the therapeutic effects of quercetin and *G. biloba* leaf extract on ACD *in vivo* and clarified the mechanism of treatment with ACD.

MATERIALS AND METHODS

Cell culture and measurement of cytotoxicity and inflammation levels

P815 cells (mouse mast cell) were purchased from the Chinese Academy of Medical Sciences (CAMS, Shanghai, China). DMEM (cat# Invitrogen 11960044) was supplemented with Glutamax (cat# Invitrogen 35050061), nonessential amino acids (cat# Invitrogen 11140050, 100 $\times$), 100 mM sodium pyruvate solution (cat# Invitrogen 11360070) and 10% newborn calf serum (Ausbio). P815 cells were incubated in disposable T75 culture flasks (Corning, USA) at 37 °C in a humidified atmosphere of 5% CO₂. Cells were grown to 90%-100% confluence.

A total of 5000/well P815 cells were seeded in 100 μ L of DMEM growth medium in 96-well plates to detect cell viability. After the cells were treated with quercetin at concentrations of 5 μ g/mL, 10 μ g/mL, 20 μ g/mL, 30 μ g/mL, 40 μ g/mL, 50 μ g/mL and 60 μ g/mL for 6 h. The medium in each well was replaced with 90 μ L fresh medium mixed with 10 μ L CCK-8 (Biosharp, China). While cells cultured with medium were set as the control group, medium alone was set as the blank group. The absorbance values were measured at 450 nm with gentle shaking before reading. We calculated the cell viability using the absorbance of cells as follows: [(As-Ab)/(Ac-

Ab)] $\times$ 100% (As, experimental group; Ac, control group; Ab, blank group).

A total of 1×10^6 /well P815 cells were seeded in 2 mL DMEM growth medium in 6-well plates to detect cytokine levels. After the cells were treated with quercetin for 3 h, the culture medium was removed, 0.5 μ g/mL C48/80 was added (Sigma, USA), and the cells were cultured for 30 min. The cells were put on ice to terminate the reaction, the supernatant was collected, and hexosaminidase (β -Hex), HIS, interleukin 4 (IL4, IL-4) and tumor necrosis factor- α (TNF- α) levels were detected with ELISA kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's recommendations.

Animals and groups

A total of 90 specific pathogen-free (SPF) 20 ± 2 g BALB/c male mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (China, permission number: SCXK 2017-0005). The environmental temperature was maintained at 20~26 °C, the relative humidity was 40% ~ 70%, and a 12 h light-dark cycle was used. The feed and drinking water were sterilized and replaced three times a week.

After a week of acclimatization, the mice were divided into 9 groups (5 per cage): (1) BC group (blank control, n=10); (2) MC group (model control, 0.3 mg/kg DNCB, n=10); (3) PC group (positive control, loratadine, n=10); (4) LQ group (2 g/kg quercetin, n=10); (5) MQ group (4 g/kg quercetin, n=10); (6) HQ group (6 g/kg quercetin, n=10); (7) LGB group (2 g/kg *G. biloba* leaf extract, n=10); (8) MGB group (4 g/kg *G. biloba* leaf extract, n=10); and (9) HGB group (6 g/kg *G. biloba* leaf extract, n=10). Quercetin and *G. biloba* leaf extract were made into feed according to different doses, and the experimental period was one month. Body weight, food intake, and rearing activity were determined every week.

The mice were fasted for 12 h with free access to water before blood collection. After one month, blood was collected from the orbit. Blood was centrifuged at 3,000 rpm for 8 min, and the supernatant was collected. The contents of IL-6 (interleukin-6), IL-4 (interleukin-4), IL-1 β (interleukin-1 β), TNF- α (tumor necrosis factor- α), IFN- γ (interferon- γ), and IgE (immunoglobulin E) were measured using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Ear swelling inhibition rate and immune organ index

On the 10th day, the mice were killed by inhalation of CO₂. After both auricles were removed, ear slices were collected with a 0.8 cm punch and weighed. The degree of ear swelling and the inhibition rate of ear swelling were

calculated according to the following formulas:

Degree of ear swelling = quality of sensitized ear slice - quality of nonsensitized ear slice

Inhibition rate of ear swelling = (degree of ear swelling in MC group - degree of ear swelling in administration group)/degree of ear swelling in MC group

The thymus and spleen were weighed, and the thymus index and spleen index were calculated according to the following formulas:

Thymus index = (thymus weight (mg)/mouse weight (g)) × 10

Spleen index = (spleen weight (mg)/mouse weight (g)) × 10

Histomorphological analysis

As soon as the experimental mice were anesthetized with CO₂ and sacrificed, ear slices were excised quickly and rinsed with ice-cold sterile PBS buffer. The tissues were kept in paraformaldehyde (4%) for histological examination overnight and trimmed. After dehydration with absolute ethanol, embedding and slicing, hematoxylin staining was performed for 8 min. The sections were washed with water after eosin staining for 2 min and sealed after ethanol gradient dehydration. The integrity of the auricular tissue, whether there was intracellular and the degree of dermal vasodilation and congestion, were observed under a microscope.

RT-qPCR

Total RNA was extracted from P815 cells and spleens using a FineMag animal tissue RNA kit (GENFINE, MR201) according to the manufacturer's recommended protocols. The integrity and concentration of RNA were separately assessed through 1.0% agarose gel electrophoresis and analysis with a NanoDrop 2000 (Thermo Scientific, USA). RNA was stored at -80 °C for subsequent experiments. Five hundred nanograms of RNA was reverse transcribed into first-strand cDNA using the Prime Script™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan), and the cDNA templates were stored at -20 °C until use. RT-qPCR was performed using SYBR® Premix Ex Taq™ II (TaKaRa, Japan). The RT-qPCR protocol consisted of an initial denaturation at 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. RT-qPCR was run on an Applied Biosystems 7500 thermocycler (Thermo Scientific, USA). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize target gene expression. The relative expression of each gene was calculated by the 2^{-ΔΔCT} method. Three biological replicates were performed for each group, and each biological replicate consisted of three technical replicates. All the genes and their primers are listed in Table I.

Data processing and statistical analysis

All of the experimental data were collected from different individuals as biological replicates. Data are expressed as the mean ± standard deviation (S.D.). Statistical analyses and the generation of graphics were performed with SPSS 22.0 software (SPSS Inc., Chicago, IL) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Student's t test or one-way ANOVA was used to assess the significance of differences between the groups, and *P* < 0.05 was considered statistically significant.

Table I. Primers of amplifying genes in P815 cells ad mice experiment.

Genes	Primers sequence (5' - 3')	bp
<i>GAPDH</i>	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA	123
<i>IL-4</i>	F: GGTCTCAACCCCCAGCTAGT R: GCCGATGATCTCTCTCAAGTGAT	102
<i>TNF-α</i>	F: CTGAACTTCGGGGTGATCGG R: GGCTTGTCACCTCGAATTTTGAGA	122
<i>Pik3r3</i>	F: AAAGCCACCTAAGCCAATGAC R: GGCATGTCCCGCAATTTGT	132
<i>Akt2</i>	F: GGCCCTGACCAGACCTTA R: GATAGCCCGCATCCACTCTTC	181
<i>Gsk-3β</i>	F: TGGCAGCAAGGTAACCACAG R: CGGTTCTTAAATCGCTTGTCCTG	189
<i>TLR4</i>	F: TTTGACACCCCTCCATAGACTTCA R: GAAACTGCAATCAAGAGTGCTG	114
<i>NF-κB1</i>	F: GGGGCCTGCAAAGGTTATC R: TGCTGTTACGGTGCATACCC	112

RESULTS

Cytotoxicity and inflammation levels in P815 cells

As shown in Figure 1, after treatment with quercetin at different concentrations for 6 h, quercetin had no significant effect on cell viability compared with the control treatment (*P* > 0.05). We selected three different concentrations (10, 20 and 30 μg/mL) of quercetin for subsequent experiments.

The inflammation levels in the P815 degranulation model are shown in Figure 2. Compared with those in the control group, the secreted levels of β-HEX, HIS, IL-4, and TNF-α significantly increased (*P* < 0.05) after P815 cells were stimulated with C48/80, which demonstrated that the P815 cell degranulation model was successfully established. However, the levels of inflammatory

mediators (β -Hex, HIS) and inflammatory cytokines (IL-4, TNF- α) significantly decreased ($P < 0.05$) after the quercetin intervention at concentrations of 10, 20 and 30 $\mu\text{g/mL}$, which suggested that quercetin can ameliorate P815 degranulation. Of note, 10 $\mu\text{g/mL}$ quercetin had a relatively better therapeutic effect on P815 degranulation.

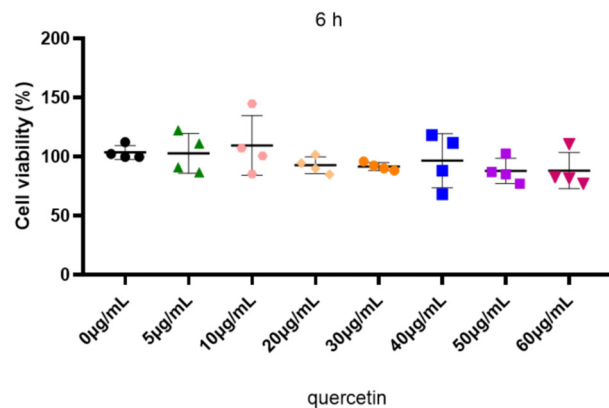


Fig. 1. Effects of different concentrations of quercetin on the cell viability of P815 cells. Data are expressed as the mean $\pm$ SD ($n = 4$).

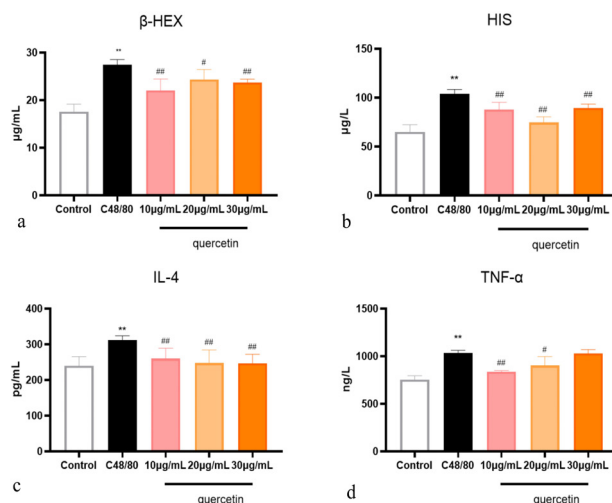


Fig. 2. Effects of quercetin on the inflammation levels in P815 degranulation model. Data are expressed as the mean $\pm$ SD ($n = 6$); * $P < 0.05$, ** $P < 0.01$ vs. Control; # $P < 0.05$, ### $P < 0.01$ vs. C48/80. β -Hex, Beta-hexosaminidase; HIS, histamine; IL-4, interleukin 4; TNF- α , tumor necrosis factor- α .

Inflammation-related mRNA expression levels in P815 cells

As shown in Figure 3, quercetin had a different influence on inflammatory gene expression. C48/80 led

to high expression of IL-4 and TNF- α , with a significant difference ($P < 0.05$) compared with the control group. In contrast, IL-4 and TNF- α levels significantly decreased after quercetin treatment at 10, 20 and 30 $\mu\text{g/mL}$ ($P < 0.05$). Although no obvious differences in Pik3r3, Akt2 and Gsk-3 β mRNA levels were detected after C48/80 stimulation ($P > 0.05$), quercetin significantly reduced gene expression to varying degrees ($P < 0.05$).

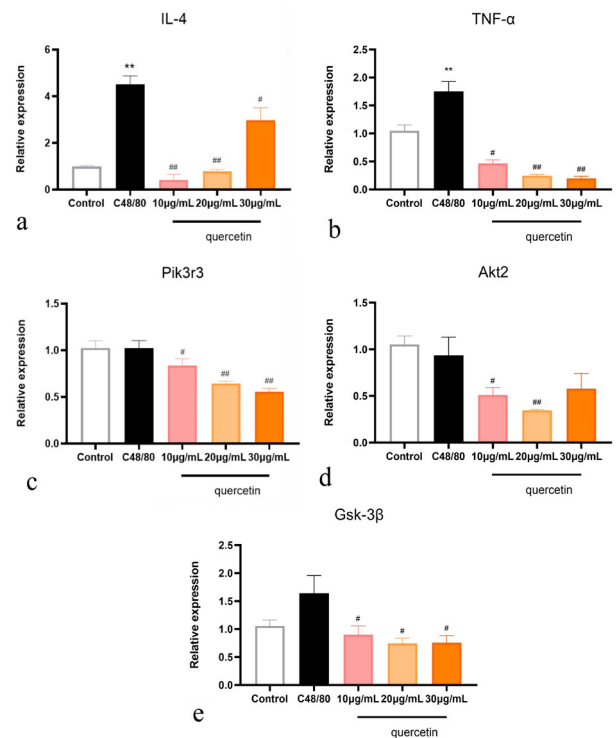


Fig. 3. Effect of quercetin on the relative expression of inflammation related mRNA in P815 degranulation model. Data are expressed as the mean $\pm$ SD ($n = 6$); * $P < 0.05$, ** $P < 0.01$ vs. Control; # $P < 0.05$, ### $P < 0.01$ vs. C48/80. Pik3r3, phosphoinositide-3-kinase regulatory subunit 3; Akt2, RAC-beta serine/threonine-protein kinase; GSK-3 β , glycogen synthase kinase-3 beta. For other abbreviations see Figure 2.

Serum biochemical indexes in ACD mice

We analyzed inflammation- and immunity-related serum indexes after quercetin and *G. biloba* leaf extract treatment. A significant increase ($P < 0.05$) in inflammatory factor secretion by ACD mice was observed in the MC group compared with the BC group (Fig. 4), indicating that DNCB stimulated ACD. Quercetin and *G. biloba* leaf extract treatment significantly reduced IL-4, IL-1 β , IFN- γ , and IgE (Fig. 4a, c, e, f) levels at all three concentrations compared with the MC group ($P < 0.05$). The secretion

of IL-6 and TNF- α in the LQ, HQ, LGB, MGB and HGB groups was significantly decreased compared with that in the MC group ($P < 0.05$).

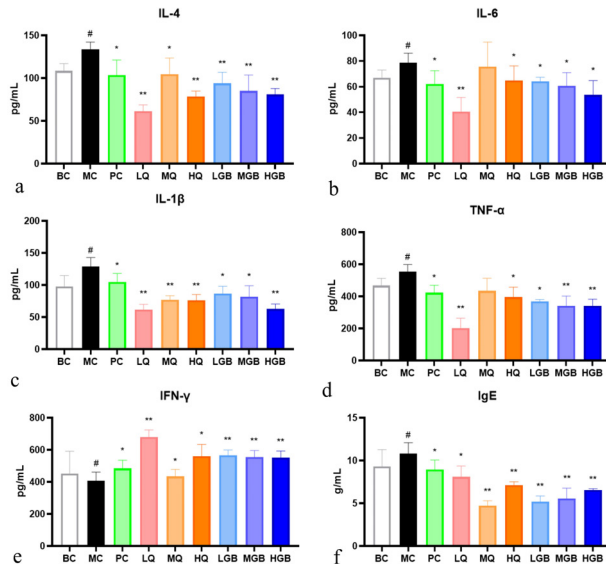


Fig. 4. Effects of different doses of quercetin and *G. biloba* leaf extract on serum biochemical indexes in ACD mice. Data are expressed as the mean $\pm$ SD ($n = 8$); $^{\#}P < 0.05$, $^{##}P < 0.01$ vs. BC group; $^*P < 0.05$, $^{**}P < 0.01$ vs. MC group. IFN- γ , Interferon gamma; IgE, immunoglobulin E. BC, blank control; MC, model control (0.3 mg/kg DNCB); PC, positive control (loratadine); LQ, 2 g/kg quercetin; MQ, 4 g/kg quercetin; HQ, 6 g/kg quercetin; LGB, 2 g/kg *G. biloba* leaf extract; MGB, 4 g/kg *G. biloba* leaf extract; HGB, 6 g/kg *G. biloba* leaf extract. For other abbreviations see Figure 2.

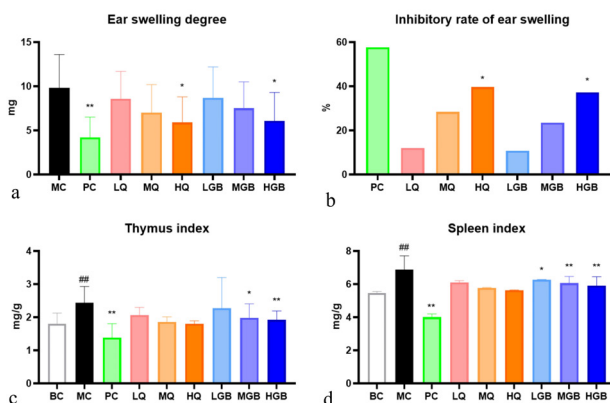


Fig. 5. Comparison of the thymus index and spleen index in each group. Data are expressed as the mean $\pm$ SD ($n = 8$); $^{\#}P < 0.05$, $^{##}P < 0.01$ vs. BC group; $^*P < 0.05$, $^{**}P < 0.01$ vs. MC group. For other abbreviation see Figure 4.

Ear swelling inhibition rate and immune organ index

Compared with that of the mice in the MC group (Fig. 5), the ear swelling degree of the mice in the PC, HQ and HGB groups decreased significantly ($P < 0.01$), while the ear swelling of mice in the other groups showed a decreasing trend. The results showed that quercetin and *G. biloba* leaf extract could relieve ear swelling in the ACD mouse model, and the dose of quercetin and *G. biloba* leaf extract was positively correlated with the inhibition rate of mouse ear swelling.

As shown in Figure 5, the thymus index and spleen index of the MC group were significantly higher than those of the BC group ($P < 0.01$). Compared with those in the MC group, the thymus index in the PC, MGB and HGB groups significantly decreased ($P < 0.05$), and the spleen index in the PC, LGB, MGB and HGB groups significantly decreased ($P < 0.05$). The results indicated that quercetin and *G. biloba* leaf extract could reduce the thymus and spleen indexes, and the dose was negatively correlated with the thymus and spleen indexes.

Histopathological results

The pathological morphology of ear slices from mice in each group, as revealed by micrography, is shown in Figure 6. There was no intercellular or intracellular swelling in the BC group, and there were no obvious pathological abnormalities. In contrast, abundant neutrophils and eosinophils infiltrated the MC group, epidermal cells ruptured, endothelial cells swelled, and blood vessels dilated. In the PC group, the skin tissue structure was intact, a small number of inflammatory cells infiltrated the spinous layer and basal layer, and the vascular endothelial cells were slightly swollen. The above results showed that the ACD model induced by DNCB was successfully established. The PC group was the positive control group, and the swelling of the tissue structure was obviously alleviated, and the inflammatory cells were reduced.

In addition, the number of inflammatory cells in the HQ and HGB groups decreased to varying degrees, and skin injury and tissue swelling decreased, indicating that quercetin and *G. biloba* leaf extract have good therapeutic effects on the ACD mouse model.

Inflammation-related mRNA expression levels in mouse experiments

The qPCR results are shown in Figure 7. The mRNA expression levels of TLR4, NF- κ B1, and TNF- α in the MC group increased significantly compared with those in the BC group ($P < 0.01$). However, inflammation-related mRNA expression levels in the PC group decreased significantly compared with those in the MC group ($P < 0.01$). Similarly, different doses of quercetin and *Ginkgo biloba* leaf extract

downregulated the mRNA expression levels of TLR4, NF- κ B1, and TNF- α . Compared with that in the MC group, the expression of TLR4 decreased significantly in the HQ and HGB groups ($P < 0.05$), and the expression of NF- κ B1 decreased significantly in the MPG group ($P < 0.05$). In addition, the expression of TNF- α decreased significantly in the MQ, HQ, and HGB groups ($P < 0.05$).

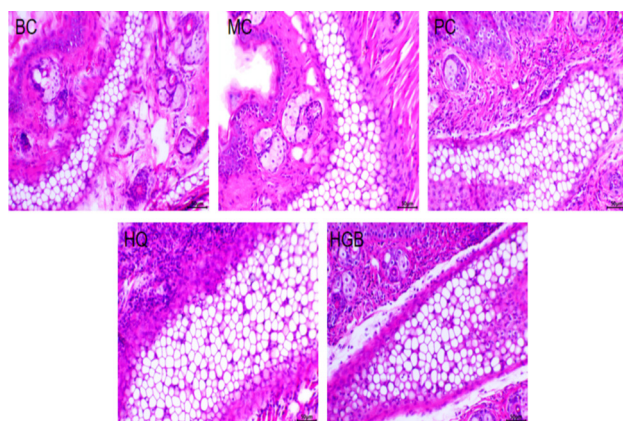


Fig. 6. Histopathological micrographs of ear slices of mice in each group (X200). For other abbreviation see Figure 4.

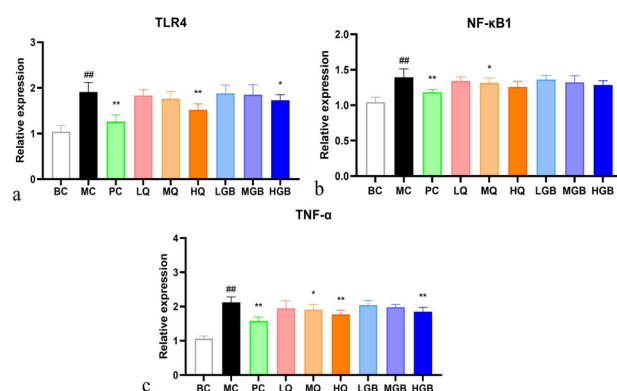


Fig. 7. Relative expression of inflammation related genes in mouse. Data are expressed as the mean $\pm$ SD ($n = 8$); # $P < 0.05$, ## $P < 0.01$ vs. BC group; * $P < 0.05$, ** $P < 0.01$ vs. MC group. For other abbreviation see Figure 4.

DISCUSSION

Mast cells are involved in acute and chronic allergic reactions as primary effector cells. They can affect subsequent allergic reactions by initiating or aggravating inflammatory reactions. β -HEX, HIS and inflammatory factors are released after mast cell degranulation, hence activating endothelial cells and further directly or indirectly leading to telangiectasia and dermal edema

(Fukuishi *et al.*, 2014). β -HEX is the main mediator in mast cell degranulation, which is positively correlated with HIS. HIS is widely present in the mucosa of human tissues and is activated and released in a free state if the body is injured (Falcone *et al.*, 2017). IL-4 and TNF- α play an indispensable role in allergic reactions and cause various pathophysiological characteristics (Jacobsson and Fowler, 2001). In our study, C48/80 was successfully used to induce the mast cell activation model *in vitro*. The experimental results showed that 10, 20, and 30 μ g/mL quercetin significantly reduced the levels of β -HEX, HIS, IL-4, and TNF- α in mast cells.

PCR experiments further proved that quercetin could regulate the mRNA expression levels of IL-4, TNF- α , Pik3r3, Akt2 and Gsk-3 β . Studies have proven that sesamin effectively inhibits the release of inflammatory cytokines from mast cells by inhibiting the PI3k/Akt signaling pathway (Li *et al.*, 2015). The PI3k/Akt signaling pathway is closely related to cell growth, cell proliferation and cell differentiation regulatory factors, which generally participate in cell cycle activities. PI3K is a pivotal molecule connecting intracellular and extracellular signals and cellular response effects, which can be activated by many upstream or bypass signal molecules (Physiol *et al.*, 2010). Pik3r3 is a regulatory subunit of PI3k phosphoinositide-3-kinase associated with a variety of inflammatory diseases, lung cancer and type II diabetes (Lin *et al.*, 2012). Some studies have found that the activation of Gsk-3 β may be related to the production of inflammatory factors and the occurrence and development of cancer (Wang *et al.*, 2013).

ACD is an inflammatory skin disease caused by delayed allergic reactions mediated by T cells (Sather *et al.*, 2007). At present, the treatment of ACD mainly includes oral antihistamines or glucocorticoids and humectants for external use (Tocci *et al.*, 2015). The pathological process of ACD involves Th1 and Th2 cells and a variety of cytokines, which are mainly produced by Th1 cells (Neis *et al.*, 2006). Th1-type cells mainly secrete TNF- α and IFN- γ , and Th2-type cells mainly secrete IL-4 and IL-6, which jointly affect the occurrence, development and prognosis of the ACD pathological process (Scala *et al.*, 2006). Increased levels of anti-inflammatory factors (IL-4, IL-10, etc.) can inhibit the ACD response, and proinflammatory factors (IL-6, IL-1 β , TNF- α , IFN- γ , IgE) can aggravate the ACD response (Chen *et al.*, 2017; Li *et al.*, 2008). As several studies have demonstrated, traditional Chinese herbal medicines have natural ingredients as well as low cytotoxicity, and the mechanism of their anti-allergic effects are multitarget and multipathway. Quercetin and *G. biloba* leaf extract can inhibit serum inflammatory cytokines in the ACD mouse model, indicating that quercetin and *G. biloba* leaf extract have a specific inhibitory effect on ACD.

The spleen and thymus are important immune organs of the body, and their quality can reflect the strength of immune function. The spleen participates in humoral and cellular immunity and can produce T cells, B cells, macrophages and so on (Al *et al.*, 2006). The thymus participates in cellular immunity and can primarily produce T cells. In this experiment, the indexes of the spleen and thymus in the *G. biloba* leaf extract administration groups showed a significant decrease. *G. biloba* leaf extract might relieve the body's allergic reaction by inhibiting the excessive differentiation of T cells, B cells and macrophages. Histopathological results also showed that quercetin and *G. biloba* leaf extract have good anti-inflammatory effects.

Inflammatory and immune responses occur throughout the pathological process of ACD. TLR4, a vital member of the Toll-like receptor family, plays a critical role in inflammatory and immune responses (Yuan *et al.*, 2018). NF- κ B1, a key nuclear transcription factor in ACD, can activate proinflammatory genes and regulate inflammatory cytokines (Kim *et al.*, 2019). Inhibition of the TLR4/NF- κ B inflammatory signaling pathway is of great significance for alleviating ACD. After interventions with quercetin and *G. biloba* leaf extract, the expression of TLR4, NF- κ B1, and TNF- α mRNA decreased in a dose-dependent manner, indicating that proinflammatory cytokines were inhibited.

CONCLUSION

As stated above, quercetin is slightly more effective than *G. biloba* leaf extract in the treatment of ACD. The mechanism may involve reducing proinflammatory cytokine levels and regulating the TLR4/NF- κ B signaling pathway to inhibit the inflammatory response and improve immune dysfunction. Quercetin is the main active ingredient of *G. biloba* leaf extract, and it plays an anti-inflammatory and anti-allergic role.

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IRB approval

All experiments performed on animals were approved by the Animal welfare and Ethics Committee of Zhejiang Academy of Agricultural Sciences (Ethics protocol no. 1935) in accordance with the Chinese guidelines for the care and use of laboratory animals. Animal studies were conducted following the principles and guidelines of the Farm Animal Welfare Council of Zhejiang, China.

Ethical statement

This study was approved by the Ethics Committee of Zhejiang Academy of Agricultural Sciences (Ethics protocol no. 1935).

Statement of conflicts of interest

The authors have declared no conflict of interest.

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