



Immunomodulatory effect of thymoquinone on atopic dermatitis

Hina Aslam^{a,d}, Muhammad Shahzad^{a,*}, Arham Shabbir^{a,b}, Sabeen Irshad^{a,c}

^a Department of Pharmacology, University of Health Sciences, Lahore, Pakistan

^b Department of Pharmacy, The University of Lahore-Gujrat Campus, Gujrat, Pakistan

^c Department of Pharmacology, Allama Iqbal Medical College, Lahore, Pakistan

^d Department of Pharmacology, King Edward Medical University, Lahore, Pakistan

ARTICLE INFO

Keywords:

Atopic dermatitis
Thymoquinone
Immunomodulation
IgE
IL-5
IFN- γ

ABSTRACT

Background: Atopic dermatitis (AD) or atopic eczema is a skin disease characterized with itching, increased serum level of immunoglobulin E, and peripheral eosinophilia. Thymoquinone (TQ) is an important ingredient of *Nigella sativa* seeds having antioxidant and anti-inflammatory effects.

Objective: Present study investigates the immunomodulatory effects of Thymoquinone (TQ) in mice model of atopic dermatitis.

Methods: Ear pinnas of mice were sensitized and challenged with DNCB (2–4 di nitro chloro benzene) to induce AD-like lesions. The mice were then treated with TQ and tacrolimus, both orally and topically. Ear thickness and weight were measured along with gross changes. Total and differential leukocyte counts were measured in blood. Total serum IgE levels were measured by enzyme linked immunosorbent assay (ELISA). The mRNA expression levels of IL-4, IL-5, and IFN- γ in ear tissue were measured using reverse transcription polymerase chain reaction (RT-PCR).

Results: Both oral and topical thymoquinone showed the potential to improve atopic dermatitis by significantly reducing the inflammatory cells infiltration in the blood ($p < 0.001$) and improving the dermatitis score ($p < 0.001$). Significant reduction in ear thickness ($p < 0.001$) and IgE levels ($p < 0.001$) were also observed. TQ and tacrolimus also significantly attenuated mRNA expression levels of IL-4, IL-5 and IFN- γ ($p < 0.001$).

Conclusions & clinical relevance: Taken together, our results showed that oral and topical application of thymoquinone exerts immunomodulatory effects in animal model of atopic dermatitis, suggesting further studies and clinical trials to establish it as a candidate nutraceutical for the treatment of AD.

1. Introduction

Atopic dermatitis or atopic eczema is a continuous itchy state characterized by inflammation which generally starts in infancy and extends into adult life. The disease is genetically susceptible and its expressions are altered by environmental factors. The distinctive characteristics of AD are frequent occurrence, reverting and worsening of condition, a disrupted state of epidermal-barrier function that end up in skin dryness, and IgE-mediated changes to various food and environmental irritant substances (Abdel-Hamid, 2003). AD is considered as biphasic disorder and is also characterized by disequilibrium between Th1/Th2 lymphocytes (Agrawal et al., 2011). The reactions to irritants are elicited by Th2 immune response, while infections are elicited by Th1 immune responses (Romagnani, 2000). IL-4, IL-5, and IL-13 are representative Th2 type cytokines which stimulate the differentiation of Th2 cells and IgE generation by B cells. Whereas, IL-12 and IFN- γ are

representative Th1 type cytokines that cause the differentiation of T cells into Th1 type cells (Horikawa et al., 2002).

There are different treatment options available to balance the disequilibrium state in AD, but the success rate is very low. The management does not completely relieve AD, and is not free of adverse effects as well. Treatment over time is also affected by genetic variations in FLG and TSLP genotype (Chang et al., 2017).

Topical calcineurin inhibitors are chosen as second-line drugs in patients above 2 year age. They are used as a substitute to topical corticosteroids and are recommended for the limited time. They are also prescribed for off and on chronic treatment in moderate to severe cases of AD (Krakowski et al., 2008). Tacrolimus, a calcineurin inhibitor, is usually suggested to those patients who stop responding to steroids, or due to steroid induced atrophy and various adverse effects. It is an immunosuppressive drug and suppresses the production of associated cytokines (Yamamoto and Nishioka, 2003). The adverse effects

* Corresponding author at: Department of Pharmacology, University of Health Sciences, Kahayan-e-Jamia Punjab, Lahore, Pakistan.

E-mail addresses: shahzad912@hotmail.com, shahzad912@uhs.edu.pk (M. Shahzad).

noticed from the use of tacrolimus include burning sensation and itching on the application spot. Some studies also reported malignancy (i.e., skin cancer and lymphoma) its use (Leung and Bieber, 2003).

Nigella sativa is an attractive alternative for the treatment of allergic/atopic diseases (Badary et al., 2000). Its use in eczema has been accepted worldwide (Goreja, 2003). Many studies showed that the biological activity of *Nigella sativa* seeds were primarily associated with thymoquinone, an essential oil component of plant (Hajhashemi et al., 2004; Burits and Bucar, 2000; Gali-Muhtasib et al., 2006). TQ possesses various antioxidant and anti-inflammatory properties. It is also known to reduce the incidence of arthritis (Budancamanak et al., 2006; Badary et al., 2003; Tekeoglu et al., 2007). It has exhibited positive effects in different clinical conditions including protection against skin inflammation (Kundu et al., 2013). The present study investigates the immunomodulatory effects of TQ on AD-like lesions using BALB/c mice model.

2. Material and methods

2.1. Animals

A total of 60 healthy female BALB/c mice, weighing 30–35 g, were divided into six groups and each group comprised of ten mice. The animals were kept under standard environmental conditions in the Experimental Research Laboratory of UHS Lahore at controlled room temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$), and light/darkness conditions. The animals were fed on standard diet and water *ad libitum*. The study was approved by Ethical Review Committee (Pharma/UHS/204) and Advanced Studies and Research Board of University of Health Sciences, Lahore.

2.2. Experimental design

Animals were numbered from 1 to 60 and assigned randomly to groups I, II, III, IV, V, and VI. The groups were then designated as control, diseased, and experimental groups.

2.3. Drugs and chemicals

Tacrolimus was provided from CCL Pharmaceuticals (Lahore, Pakistan). DNCB and thymoquinone ($\geq 98\%$) were purchased from Sigma Aldrich (US).

2.4. Induction of AD-like skin lesions in the ear

DNCB was used to induce AD-like skin lesions according to the method of Chan et al. (2013) with modification. To induce AD-like skin lesions in diseased and treatment groups, mice were sensitized by applying 20 μl of 0.5% DNCB in acetone / olive oil (3:1) on pinna of each ear at days 1–3. Two weeks later, mice in diseased and treatment groups were challenged by applying 20 μl of 1% DNCB on pinna of each ear at days 14, 17, 20, 23, 26 and 29. These groups were also treated with TQ and tacrolimus during days 14–29.

2.5. Group I (control)

The mice were sham-sensitized and challenged epicutaneously on pinna of each ear with acetone/olive oil (3:1). Ethanol/distilled water (3 ml) in 1:2 ratio was also given orally, one hour before the challenge.

2.6. Group II (diseased)

The mice were sensitized at days 1–3 and then six times epicutaneously challenged starting from day 14 to day 29 on pinna of each ear with DNCB.

2.7. Group III (experimental I/ oral tacrolimus)

The mice were treated with tacrolimus (30 mg/kg body weight) orally dissolved in 3 ml ethanol/distilled water (1:2), one hour before the challenge (Meingassner et al., 2003).

2.8. Group IV (experimental II/ topical tacrolimus)

The mice were treated with tacrolimus (1%) topically dissolved in ethanol (0.2 mg of tacrolimus was dissolved in 0.02 ml of ethanol), one hour before challenge (Hirotaka et al., 2010).

2.9. Group v (experimental III/oral thymoquinone)

The mice were treated with TQ (10 mg/kg body weight) orally, dissolved in 3 ml ethanol/distilled water (1:2), one hour before the challenge (Ammar et al., 2011).

2.10. Group VI (experimental IV/ topical thymoquinone)

The mice were treated with TQ (5 μmol in 0.2 ml acetone), one hour before the challenge. The picked dose was based on the results of Kundu et al. (2013), who used same dose of TQ to elucidate the molecular mechanism of anti-inflammatory activity TQ in mouse skin.

All animals were sacrificed 24 h after the last treatment and challenge at day 30 under light ether anesthesia. Blood sample was collected to measure TLC and DLC. Serum was stored at -20°C to measure IgE levels. Ears were excised to measure weight and determine mRNA expression level of cytokines.

2.11. Assessment of the ear clinical score/ macroscopic evaluation of ear skin lesions

Clinical symptoms of each mouse were evaluated at day 0, 14 and 29. Briefly, erythema, edema, excoriation and dryness on the ear surface were scored as 0 (not visible), 1 (mild), 2 (moderate) and 3 (severe). Scoring was performed by two independent observers, and the final score was taken as an average for each group (Matsuoka et al., 2003). Skin status of each group was also photographed before and after the treatment.

2.12. Ear thickness

Right and left ear thickness was measured at day 0, 14 and 29, with a dial thickness gauge, before challenge and treatment. For each mouse, both right and left ears were measured, and an average was calculated.

2.13. Analysis of hematological parameters

TLC and DLC in blood were determined by microscopic evaluation. The blood was collected 24 h after the last treatment and challenge in EDTA vacutainer via cardiac puncture at day 30. For TLC, the diluted blood was stained with gentian violet and glacial acetic acid. Then the blood was transferred to Neubauer counting chamber and cells were counted under low power microscope. For DLC, diluted blood was stained with Wright-Giemsa stain and counted under microscope.

2.14. Ear weight

The excised right and left ear was weighed soon after they were sacrificed. The difference of weight represented the severity of inflammation.

2.15. Determination of IgE levels by ELISA

For determination of IgE levels, blood was collected in serum

Table 1
Primer sequences.

Sr/No	Primers	Sequence
1.	IL-4	5-TCACTGACGGCAGAGCTA-3 5-CCTTCTCCTGTGACCTCGTT-3 NCBI Reference sequence: NM 021283.2
2.	IL-5	5-GGCTGGCCTCAAACTGGTAA-3 5-CCCTGATGCAACGAAGAGGA-3 MGI:96557
3.	IFN- γ	5-CCATCGGCTGACCTAGA-3 5-GCCACTTGAGTTAAATAGTTATTTCAGAC-3 (Harris et al., 2005)

separator tubes. Sera were separated by centrifugation and stored at -20°C . IgE levels were measured by ELISA according to kit manufacturer's instruction (Glory Science Co., Ltd.). An optical density was measured at 450 nm wavelength.

2.16. Determination of mRNA expression levels of IL-4, IL-5 and IFN- γ by reverse transcription polymerase chain reaction (RT-PCR)

For determination of cytokine expression levels, total RNA from ear tissue was extracted by TRIzol method (Shabbir et al., 2014). cDNA was synthesized by reverse transcription by following using kit manufacturer's protocol (Thermo Scientific, America). The cDNA prepared by reverse transcription was amplified by PCR using gene specific primers. For amplification of cDNA, 10 μl PCR pre mix, 1 μl forward primer, 1 μl reverse primer, 2 μl cDNA (from first strand reaction) and 20 μl nuclease-free water were added in a thin walled PCR tube. The mixture was placed in a thermal cycler with 32 cycles of denaturation (95°C), annealing (58°C), and termination (72°C). 6 μl of PCR product was

loaded on 1.5% Agarose gel. PCR product was visualized through gel documentation system. Image J software was used for semi-quantification of PCR product. Primers used in cDNA synthesis are shown in Table 1. Oligonucleotide primers for IL-4, IL-5 and IFN- γ were purchased from Thermo Scientific, America.

2.17. Statistical analysis

Data were entered and analyzed by using the GraphPad Prism v.6. Mean $\pm$ Standard deviation (SD) was given for normally distributed quantitative variables. One way ANOVA was used to observe the differences in groups. *Post hoc* Tukey's test was also applied to determine the difference among different groups. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Effect of TQ on blood inflammatory cells

Two weeks after successful induction of atopic dermatitis-like alterations of the immune system, oral and topical TQ treatment was able to reduce TLC levels compared to diseased group with p value ≤ 0.001 (5.500 ± 0.5345 vs 7.875 ± 0.8345) and (4.250 ± 0.7071 vs 7.875 ± 0.8345) respectively. Similarly, tacrolimus orally and topically treated group also showed a significant decrease in TLC as compared to diseased group with p value ≤ 0.001 (5.375 ± 0.7440 vs 7.875 ± 0.8345) and (4.625 ± 0.5175 vs 7.875 ± 0.8345), respectively. There was noticeable difference in TLC of topically TQ treated group compared to orally TQ treated group with p value ≤ 0.01 (4.250 ± 0.7071 vs 5.500 ± 0.5345) and oral tacrolimus vs topical TQ with p value ≤ 0.05 (5.375 ± 0.7440 vs 4.250 ± 0.7071). There

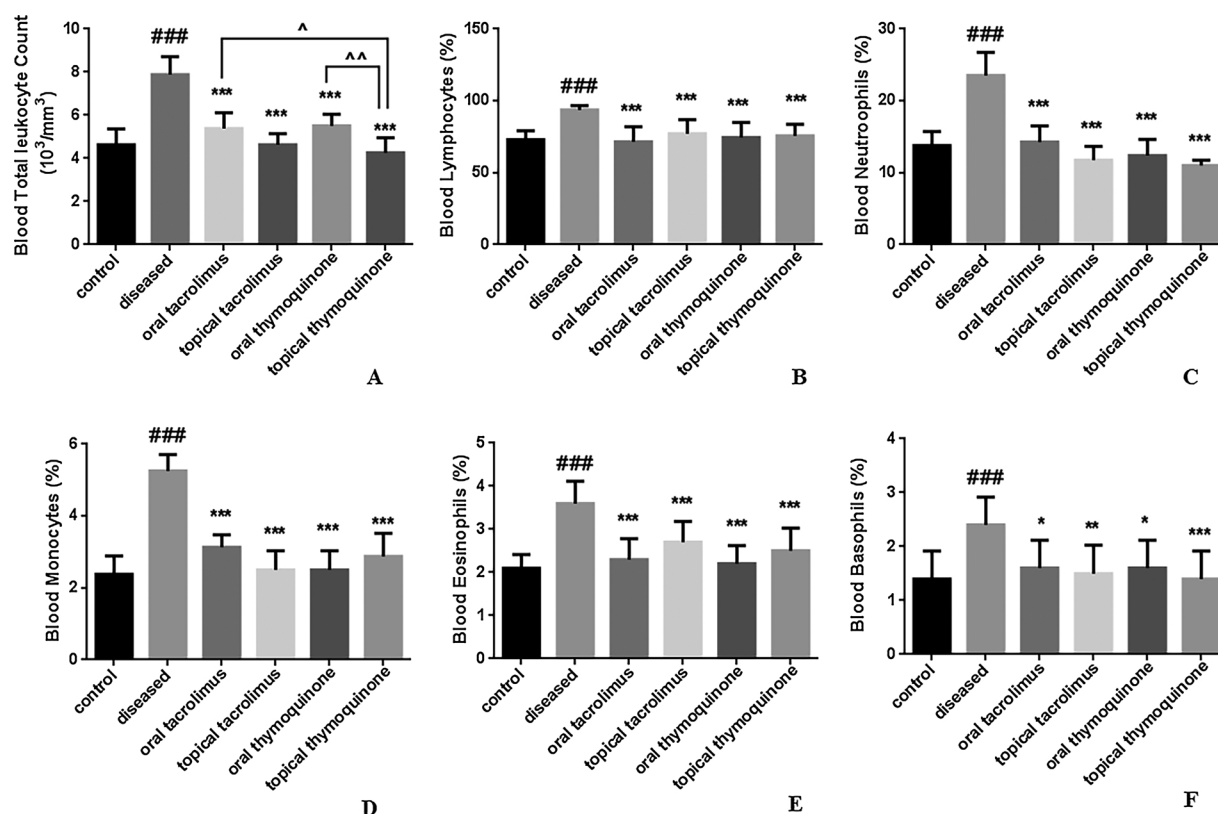


Fig. 1. Graphical representation of mean $\pm$ SD of (A) TLC (no. of leukocyte $\times 10^3 / \text{mm}^3$) (B) lymphocytes % (C) neutrophils % (D) monocytes % (E) eosinophils % (F) basophils % in blood in all groups ($n = 10$). ### shows $p \leq 0.001$ and indicates significant difference as compared to control group. *** shows p value ≤ 0.001 , ** shows p value ≤ 0.01 and * shows p value ≤ 0.05 and indicates significant difference as compared to diseased group. ^ shows p value ≤ 0.05 and ^^ shows p value ≤ 0.01 .

was no noticeable difference in TLC between other treatment groups.

DNCB sensitization and challenge resulted in the accumulation of neutrophils, lymphocytes, monocytes, eosinophils and basophils in diseased and all experimental groups. TQ orally and topically treated groups showed a significant decrease in DLC as compared to group II (diseased). Similarly, tacrolimus orally and topically treated groups showed significantly decreased levels of DLC as compared to group II (diseased). When the individual effects of TQ and tacrolimus were compared with one another, we found no significant difference between them (Fig. 1).

3.2. Effect of TQ on ear thickness

Induction of atopic dermatitis in BALB/c mice model significantly increased ear thickness in diseased group at day 14 with p value ≤ 0.001 . Treatment with TQ orally and topically was able to reduce thickness of ear significantly as compared to diseased group at day 29 (0.484 ± 0.066 vs 0.849 ± 0.064) with p value ≤ 0.001 and (0.540 ± 0.039 vs 0.849 ± 0.064) with p value ≤ 0.001 respectively. Similarly, treatment with tacrolimus orally and topically also significantly reduced the ear thickness as compared to diseased group (0.480 ± 0.048 vs 0.849 ± 0.064) with p value ≤ 0.001 and (0.498 ± 0.043 vs 0.849 ± 0.064) with p value ≤ 0.001 respectively. There was no noteworthy difference between treated groups (Fig. 2A–C).

3.3. Effect of TQ on ear clinical score

Sensitization with DNCB in BALB/c mice model significantly increased ear clinical score at day 14 in diseased group and all experimental groups with p value ≤ 0.001 . At day 29, treatment with TQ orally and topically was able to reduce clinical score of ear significantly as compared to diseased group with p value ≤ 0.001 (2.90 ± 0.73 vs

14.00 ± 1.82) and (4.40 ± 0.96 vs 14.00 ± 1.82), respectively. Similarly treatment with tacrolimus orally and topically significantly reduced the clinical score as compared to diseased group with p value ≤ 0.001 (3.00 ± 0.81 vs 14.00 ± 1.82) and (3.50 ± 0.70 vs 14.00 ± 1.82), respectively. There was significant difference in ear clinical score of topically TQ treated group compared to orally TQ treated group with p value ≤ 0.05 (4.40 ± 0.96 vs 2.90 ± 0.73) and oral tacrolimus vs topical thymoquinone (3.00 ± 0.81 vs 4.40 ± 0.96), respectively. No remarkable difference was observed between other treatment groups (Fig. 2E,F).

3.4. Effect of TQ on ear weight

Our data showed significant increase in ear weight (mg) of group II (diseased) as compared to group I (control) (92.52 ± 19.56 vs 32.23 ± 7.194) with p value ≤ 0.001 respectively. TQ orally and topically treated groups showed a significant decrease in ear weight as compared to diseased group II with p value ≤ 0.001 (54.06 ± 5.016 vs 92.52 ± 19.56) and (63.69 ± 14.20 vs 92.52 ± 19.56), respectively. Similarly tacrolimus orally and topically treated group showed significant result as compared to group II (diseased) with p value ≤ 0.001 (60.37 ± 6.445 vs 92.52 ± 19.56) and (54.53 ± 7.746 vs 92.52 ± 19.56), respectively. No any noteworthy difference was observed between treatment groups (Fig. 2D).

3.5. Effect of TQ on IgE levels

Sensitization and challenge with DNCB significantly increased IgE levels (ng/ml) of group II (diseased) as compared to group I (control) (2177 ± 331.2 vs 591.4 ± 51.37) with p value ≤ 0.001 respectively. Significantly decreased levels of IgE were observed after oral and topical treatment with TQ as compared to diseased group with p value ≤ 0.001 (812.1 ± 116.6 vs 2177 ± 331.2) and (693.6 ± 180.8 vs

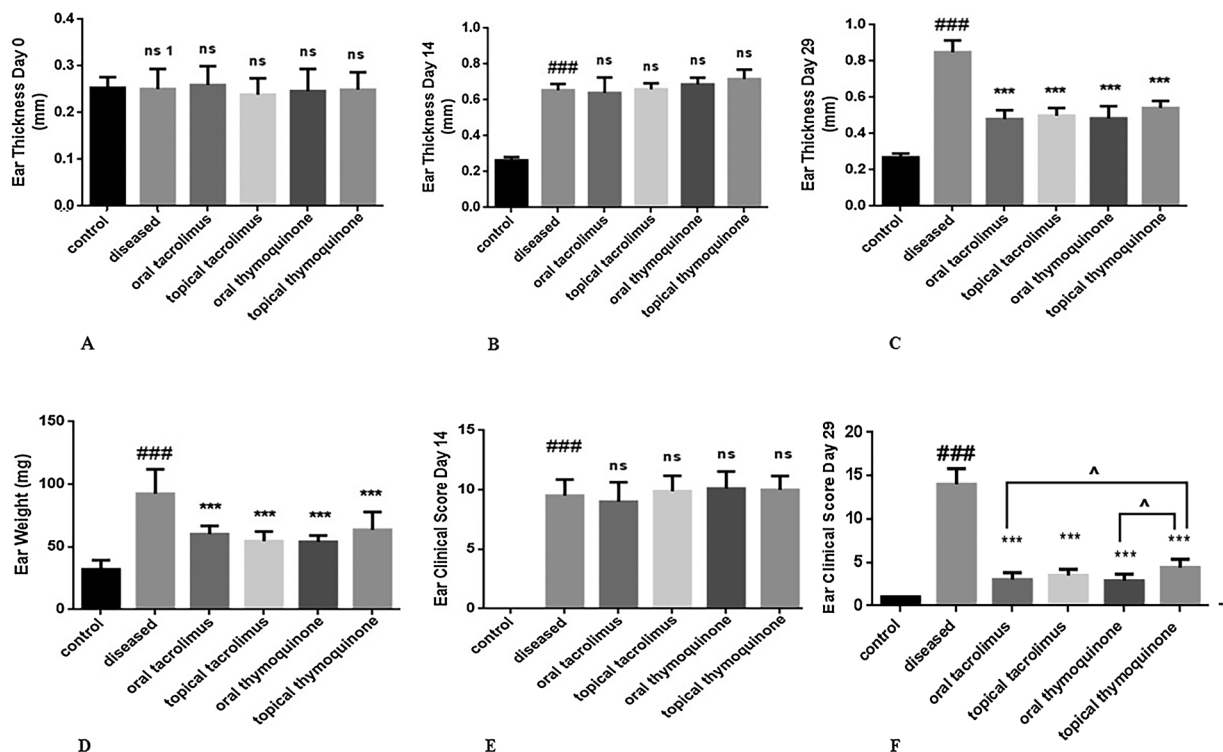


Fig. 2. Graphical representation of mean $\pm$ SD of ear thickness (mm) day 0 (A), day 14 (B), day 29 (C), ear weight (mg) (D), ear clinical score day 14 (E) and day 29 (F) in all groups ($n = 10$). ns shows no significant difference with group II. ns¹ shows no significant difference with group I. ### shows $p \leq 0.001$ and indicates significant difference as compared to control group. *** shows p value ≤ 0.001 and indicates significant difference as compared to diseased group. ^ shows p value ≤ 0.05 .

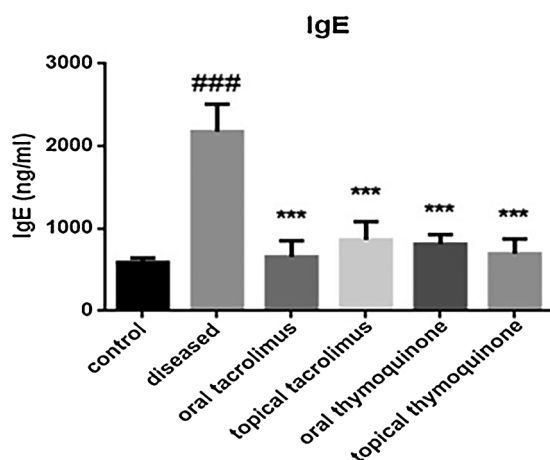


Fig. 3. Graphical representation of mean $\pm$ SD of levels of IgE in all groups ($n = 10$). ### shows $p \leq 0.001$ and indicates significant difference as compared to control group. *** shows p value ≤ 0.001 and indicates significant difference as compared to diseased group.

2177 ± 331.2), respectively. Similarly, oral and topical tacrolimus showed significant decrease in IgE levels as compared to group II (diseased) with p value ≤ 0.001 (655.7 ± 199.0 vs 2177 ± 331.2) and (863.6 ± 219.8 vs 2177 ± 331.2), respectively. When the individual effect of TQ and tacrolimus was compared with one another, there was no significant difference found between them (Fig. 3).

3.6. Effect of TQ on mRNA expression levels of IL-4, IL-5, and IFN- γ

We observed a significant increase in mRNA expression levels of IL-4, IL-5, and IFN- γ of diseased group II as compared to control group with p value ≤ 0.001 (1.890 ± 0.2724 vs 1 ± 0), (1.982 ± 0.3217 vs 1 ± 0) and (6.022 ± 1.260 vs 1 ± 0), respectively. This showed that DNCB sensitization and challenge in diseased group resulted in increased mRNA expression levels of IL-4, IL-5, and IFN- γ . TQ orally and topically treated groups showed a significant decrease in mRNA expression levels of IL-4 as compared to diseased group with p value ≤ 0.001 (1.224 ± 0.2220 vs 1.890 ± 0.2724) and (0.6814 ± 0.2347 vs 1.890 ± 0.2724), respectively. Similarly, orally and topically tacrolimus treated group also showed a significant decrease in expression levels of IL-4 as compared to diseased group with p value ≤ 0.001 (1.296 ± 0.210 vs 1.890 ± 0.2724) and (0.8614 ± 0.2204 vs 1.890 ± 0.2724), respectively. When TQ orally vs topically treated groups and oral vs topically treated tacrolimus group were compared

with each other, significant difference was observed among them (1.224 ± 0.2220 vs 0.6814 ± 0.2347) with p value ≤ 0.001 and (1.296 ± 0.210 vs 0.8614 ± 0.2204) with p value ≤ 0.01 . When the individual effects of TQ and tacrolimus were compared with each another, significant difference was observed between oral tacrolimus vs topical thymoquinone (1.296 ± 0.2106 vs 0.6814 ± 0.2347) with p value ≤ 0.001 , and topical tacrolimus vs oral thymoquinone (0.8614 ± 0.2204 vs 1.224 ± 0.2220) with p value ≤ 0.05 .

Data showed that treatment with oral and topical TQ caused a significant decrease in mRNA expression levels of IL-5 in comparison with diseased group (1.390 ± 0.1142 vs 1.982 ± 0.3217) with p value ≤ 0.01 and (1.118 ± 0.3185 vs 1.982 ± 0.3217) with p value ≤ 0.001 respectively. Similarly, orally and topically tacrolimus treated groups showed significant decrease in mRNA expression levels of IL-5 as compared to group II (diseased) with p value ≤ 0.001 (1.372 ± 0.09642 vs 1.982 ± 0.3217) and (1.135 ± 0.2732 vs 1.982 ± 0.3217), respectively. There was no noteworthy difference in expression levels of IL-5 between different treatment groups.

While analyzing mRNA expression levels of IFN- γ , TQ orally and topically treated groups showed a significant decrease in expression levels of IFN- γ as compared to diseased group with p value ≤ 0.001 (1.550 ± 0.5655 vs 6.022 ± 1.260) and (1.583 ± 0.3656 vs 6.022 ± 1.260), respectively. Also, oral and topical tacrolimus showed decreased expression levels of IFN- γ as compared to diseased group with p value ≤ 0.001 (1.610 ± 0.6524 vs 6.022 ± 1.260) and (0.7267 ± 0.3436 vs 6.022 ± 1.260), respectively. There was no significant difference between TQ and tacrolimus treated groups (Fig. 4A–C).

4. Discussion

Atopic dermatitis (AD) is a common pruritic inflammatory skin condition. It is chronic and recurrent in nature, and present mostly in urban areas. It is induced due to immunologic disturbance and other factors. It is a biphasic inflammatory skin disease aggravated by instability between Th1 and Th2 immune response (Jin et al., 2009). Even though it is not lethal, it alters the quality of life. Acute AD is always linked with the production of T helper 2 type (Th2) cytokines which represent the acute phase, while Th1 represents the chronic phase chiefly producing IFN- γ and TNF- α (Homey et al., 2006). So far, to treat AD, multiple drugs are in use, including glucocorticoids, immunomodulators, PDE inhibitors, anti-histamines, anti-metabolites and anti-infectious agents (Alvin et al., 2017). Other immunomodulator drugs which are approved by FDA are dupilumab (an interleukin IL-4 and IL-13 receptor antagonist) and nemolizumab (antibody against interleukin-31). The long term safety is neither evaluated nor confirmed

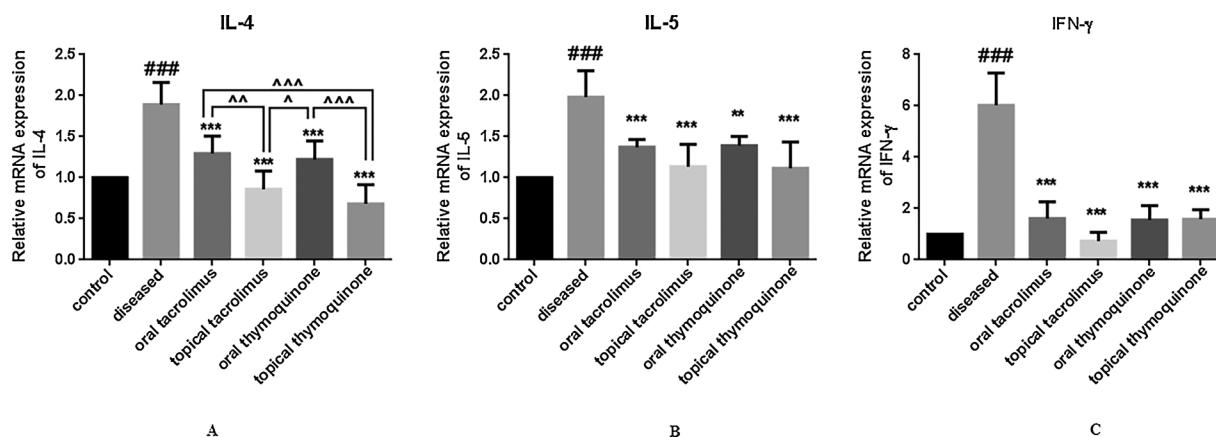


Fig. 4. Graphical representation of mean $\pm$ SD of relative mRNA expression levels of (A) IL-4, (B) IL-5 and (C) IFN- γ in all groups ($n = 10$). ### shows $p \leq 0.001$ and indicates significant difference as compared to control group. *** shows p value ≤ 0.001 , ** shows p value ≤ 0.01 and indicates significant difference as compared to diseased group. ^ shows p value ≤ 0.05 , ^^ shows p value ≤ 0.01 , and ^^^ shows p value ≤ 0.001 .

by any regulatory authority (Ofori and Corona, 2017). Until now, many natural substances have been investigated to observe anti eczematous effects (Kang et al., 2008). Atopic march is linked with three different diseases, including asthma, atopic dermatitis, and allergic rhinitis. Behind this triad, there is an interlinked chain of common pathogenesis and genetic basis along with common immunologic features. Talking about these allergic diseases, TQ significantly inhibited allergen-induced lung eosinophilic inflammation and also showed a marked reduction in IL-4, IL-5, and IgE levels in ovalbumin sensitized and challenged mice in different studies (El Gazzar et al., 2006; El Mezayen et al., 2006). TQ also ameliorated the allergic symptoms in ovalbumin induced rabbit model of allergic rhinitis (Yurttaş et al., 2016). Considering the above facts, one can hypothesize that TQ could be of therapeutic potentials in treating AD. We conducted the study to observe the immunomodulatory effects of TQ in mouse model of AD in comparison with the commonly used medicine, tacrolimus, in clinical set up. The results showed that TQ reduced all the inflammatory cells in blood, improved macroscopic changes, and downregulated the mRNA expression levels of IL-4, IL-5 and IFN- γ . To assess the immune response in the skin of animals, BALB/c mice were used in our research study. As in many studies of DNCB induced atopic dermatitis which have been conducted in BALB/c mice, the mouse model presented same scenario of AD as presented in human atopic dermatitis (Lee et al., 2010).

In our study the skins of DNCB-treated groups showed increased levels of inflammatory blood cells, raised dermatitis score, increased ear thickness, elevated IgE serum levels, and raised IL-4, IL-5, and IFN- γ expression levels than that of control groups. This chronic lesion which in addition to raised IL-4, IL-5 also showed increased expression levels of IFN- γ suggests a change over from an early Th2 response to a fused Th1 and Th2 response. A change over in time from an early Th2 to a merged Th1 plus Th2 reaction is also observed in patients with AD with house dust mite allergen (Werfel et al., 1996). These results showing a mixed Th1/Th2 response are also observed in various studies of atopic dermatitis model of BALB/c mice with the application of 2,4-dinitrochlorobenzene (DNCB) (Hwang et al., 2013; Kim et al., 2012). Acetone/olive oil used in control group cause no documented changes on the skin in the given amount (Kim et al., 2014).

It is well known that eosinophil, monocytes, neutrophils, and lymphocytes are activated in blood of AD patients (Soter, 1989; Leung and Bieber, 2003). Also the disease severity is directly linked with elevated level of eosinophils (Novak et al., 2003). And basophils take part in mediating the classical immediate-type-hypersensitivity reaction (Horsmanheimo et al., 1994). Our data indicated significant increased concentration of DLC in DNCB exposed diseased groups. On observing the TLC and DLC of the blood in all treated groups, significant reduction was seen as compared to diseased group. Previous study found increased numbers of TLC and DLC in DNCB induced mice that were found reduced to normal after treatment with *German chamomile* oil (Lee et al., 2010). Literature review also displayed that prophylactic treatment with TQ significantly improved all the disturbed values of total and differential leukocyte counts in lung lavage of sensitized guinea pigs (Keyhanmanesh et al., 2010a). The ameliorating effect on WBC counts found in this study is in line with the results of previous studies using different animal models.

However, there was noticeable difference observed in TLC of topical TQ vs oral TQ group and oral tacrolimus vs topical TQ group. No significant difference was observed between tacrolimus treated groups and oral TQ vs topical tacrolimus treated group.

Atopic dermatitis patients also present epidermal hyperplasia (Novak, 2009). In the childhood and adult phase of AD there is marked thickening of the skin (Spergel and Paller, 2003). TQ and tacrolimus treated groups remarkably reduced ear thickness in treated groups. No significant difference was observed between treated groups. Our findings are in accordance with the study of Chan et al. (2013) in which DNCB-sensitized mice treated with topical and oral DHEA (dehydroepiandrosterone) reduced the ear thickness.

Clinically, skin impairment in AD patients shows erythema, dryness, edema, and dryness (Navi et al., 2007). In our study, the animal model presented with significantly increased dermatitis score and treatment with TQ and tacrolimus displayed significant improvement in the dermatitis score of ear. Our results are in parallel with the study of Lim et al. (2014) in which the increased dermatitis score by house dust mite was decreased by *Morus Alba* L. plant. There was significant difference in ear clinical score of topical TQ vs oral TQ treated group and oral tacrolimus vs topical TQ group, whereas no significant difference was observed between other treated groups.

Likewise ear weight also shows the severity of AD. All the four treatment groups were able to decrease the ear weight significantly. Our findings are in parallel with the study of Zhou et al. (2014), in which daily dose of *Sanpaocao*, a Chinese herb, significantly reduced the ear weight in topically DNCB challenged BALB/c mice. No significant difference was seen between TQ and tacrolimus treated groups.

The elevated levels of serum IgE sensitize mast cells and enhance Th2-skewed immune responses. (Lee et al., 2010). It is also considered as the hallmark of AD (Abdel-Hamid, 2003). Our IgE data indicated significantly dropped levels in all the treatment groups. Similar results have also been reported in previous study in which, *Dendrobium tosaense*, a Chinese herbal medicine decreased the levels of IgE in AD induced by both ovalbumin and TNCB (Wu et al., 2014). In the research study of Ammar et al. (2011) administration of TQ and curcumin significantly declined the serum IgE levels compared with ovalbumin induced diseased group in asthmatic model of swiss albino mice. No significant difference was seen between TQ and tacrolimus treated groups.

In our present study, we also observed expression levels of IL-4, IL-5 and IFN- γ . IL-4 and IL-5 have the ability to stimulate B cells which then differentiate and secrete IgE (Li et al., 2013). IL-4 provokes allergic immune response and generates differentiation of naïve CD4 + T cells into TH2 cells, further releasing cytokines (Yoshimoto et al., 2000). IL-5, which is also raised in the patients with AD, contributes in the survival and differentiation of eosinophils, and in mobilization of eosinophils from bone marrow (Kondo et al., 2001; Rothenberg and Hogan, 2006). Our study displayed that TQ and tacrolimus were able to reduce increased expressions levels of IL-4 and IL-5 in treatment groups. These results are in accordance with various previously published studies. Kim et al. (2014) showed that 7, 8, 4'-Trihydroxyisoflavone, a metabolite of the soy isoflavone, daidzin, and tacrolimus attenuated DNCB-Induced AD symptoms by decreasing IL-4 and IL-5 mRNA expression levels. TQ also produced significant decrease in IL-4 and IL-5 expression levels in models of ovalbumin induced allergic airway inflammation (El Gazzar et al., 2006; El Mezayen et al., 2006; Keyhanmanesh et al., 2010b). TQ is also known to cause reduction in IL-4 secretion from allergen induced splenocytes (Gholamnezhad et al., 2015a). The anti-inflammatory and immunomodulatory effects of *N. sativa* and thymoquinone are well described in the reviews of Gholamnezhad et al. (2015b) and

Gholamnezhad et al. (2016), which further support the findings of present study. The data also showed significant difference in oral vs topical TQ group, oral vs topical tacrolimus group, oral tacrolimus vs topical TQ, and topical tacrolimus vs oral TQ.

IFN- γ produced by Th1 cells activates specific chemokines, thus causing progression towards AD lesion formation by predisposing keratinocytes to apoptosis (Ong and Leung, 2006; Trautmann et al., 2000). IFN- γ is necessary for the late phase of chronic inflammation in AD through macrophages and T cells activation (Grewe et al., 1998; Spergel et al., 1999). In our study, after treatment with TQ and tacrolimus, levels of IFN- γ expression decreased significantly. Our results are in parallel with the research study of Dang et al. (2015), which showed that treatment with C5aR antagonist declined the IFN- γ mRNA expression levels in a DNCB induced AD model of BALB/c mice. No significant difference was seen between TQ and tacrolimus treated groups.

Tacrolimus is known to possess immunosuppressive and anti-

inflammatory properties. It suppresses transcription of cytokines and inhibits T cell mediated toxicity, thus indirectly suppressing B cell growth and antibody generation which requires T cell derived growth factors for their functions. Suppression of nuclear factor- κ B was determined as one of the mechanism of anti-inflammatory property of tacrolimus (Thomson et al., 1995; Pillans, 2006; Du et al., 2009). Similar to tacrolimus, literature review showed the inhibition of expressions of various cytokines by thymoquinone in different models of inflammatory disorders. The suppression of activator protein-1/nuclear factor- κ B pathway as the potential mechanism of anti-inflammatory activity was suggested (Hossen et al., 2017). The data of current study showing inhibition of different cytokines by thymoquinone and tacrolimus is in line with the inferences of previous studies. However, effects of thymoquinone on signaling pathways in AD model require further studies.

Current study has a few limitations in terms of not testing the dose ranges of compounds under study and time course of onset of immune modulation and symptoms. Current study presented the inferences based only on mRNA expression levels of IL-4, IL-5, and IFN- γ . Previous studies have documented the presence of positive correlation between protein levels and mRNA expression levels (Greenbaum et al., 2003; Guo et al., 2008). However, in order to further strengthen the results of expression levels of these markers, future studies are suggested evaluating the protein levels of IL-4, IL-5, and IFN- γ .

5. Conclusion

To the best of our knowledge, present study is the first demonstration of the immunomodulatory effects of TQ on atopic dermatitis. The results suggested that oral and topical TQ might possess modulatory effects on Th1/Th2 immune imbalance in comparison with tacrolimus. Further studies should be carried out to find out the exact mechanism responsible for immunomodulatory effect of in AD. Our experimental results failed to indicate whether TQ or tacrolimus is more effective for the treatment of AD.

Conflict of interest

Authors declare that they have no conflict of interest.

Funding

No funding was obtained for the project by any funding agency. All the chemicals/reagents and lab facilities were used from the department of Pharmacology, University of Health Sciences, Lahore.

Acknowledgements

We are thankful to CCL pharmaceuticals for providing tacrolimus for research purpose. We are also thankful to Department of Biochemistry, Department of Hematology, Department of Immunology and Resource lab, University of Health Sciences, Lahore for providing technical facilities.

References

- Abdel-Hamid, K.M., 2003. Atopic dermatitis. In: Jost, B.C., Friedman, E., Abdel-Hamid, K.M., Jani, A.L. (Eds.), *The Washington Manual Allergy, Asthma, and Immunology Subspecialty Consult*. Lippincott Williams & Wilkins, Philadelphia, pp. 66–73.
- Agrawal, R., Wisniewski, J.A., Woodfolk, J.A., 2011. The role of regulatory T cells in atopic dermatitis. *Curr. Probl. Dermatol.* 41, 112–124.
- Alvin, W.L., Emily, S.Y., Antaya, Richard J., 2017. Topical corticosteroid phobia in atopic dermatitis. *JAMA Dermatol* [online] Available at: <http://jamanetwork.com/journals/jamadermatology/article-abstract/2643740> [Accessed 16 January 2017].
- Ammar, el-S.M., Gameil, N.M., Shawky, N.M., Nader, M.A., 2011. Comparative evaluation of anti-inflammatory properties of thymoquinone and curcumin using an asthmatic murine model. *Int. Immunopharmacol.* 11, 2232–2236.
- Badary, O.A., Abdel-Naim, A.B., Abdel-Wahab, M.H., Hamada, F.M., 2000. The influence of thymoquinone on doxorubicin induced hyperlipidemic nephropathy in rats. *Toxicology* 143, 219–226.
- Badary, O.A., Taha, R.A., Gamal el-Din, A.M., Abdel-Wahab, M.H., 2003. Thymoquinone is a potent superoxide anion scavenger. *Drug Chem. Toxicol.* 26, 87–98.
- Budancamanak, M., Kanter, M., Demirel, A., Ocakci, A., Uysal, H., Karakaya, C., 2006. Protective effects of thymoquinone and methotrexate on the renal injury in collagen-induced arthritis. *Arch. Toxicol.* 80, 768–776.
- Burits, M., Bucar, F., 2000. Antioxidant activity of *Nigella sativa* essential oil. *Phytother. Res.* 14, 323–328.
- 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. *J. Dermatol. Sci.* 72, 149–157.
- Chang, J., Mitra, N., Hoffstad, O., 2017. Association of filaggrin loss of function and thymic stromal lymphopoietin variation with treatment use in pediatric atopic dermatitis. *JAMA Dermatol.* 153 (3), 275–281.
- Dang, L., He, L., Wang, Y., Xiong, J., Bai, B., Li, Y., 2015. Role of the complement anaphylatoxin C5a-receptor pathway in atopic dermatitis in mice. *Mol. Med. Rep.* 11 (6), 4183–4189.
- Du, S., Hiramatsu, N., Hayakawa, K., Kasai, A., Okamura, M., Hang, T., Yao, J., Takeda, M., Araki, I., Sawada, N., Paton, A.W., Paton, J.C., Kitamura, M., 2009. Suppression of NF- κ B by cyclosporine a and tacrolimus (FK506) via induction of the C/EBP family: implication for unfolded protein response. *J. Immunol.* 182 (11), 7201–7211.
- El Gazzar, M., El Mezayen, R., Marecki, J.C., Nicolls, M.R., Canastar, A., Dreskin, S.C., 2006. Anti-inflammatory effect of thymoquinone in a mouse model of allergic lung inflammation. *Int. Immunopharmacol.* 6 (7), 1135–1142.
- El Mezayen, R., El Gazzar, M., Nicolls, M.R., Marecki, J.C., Dreskin, S.C., Nomiyama, H., 2006. Effect of thymoquinone on cyclooxygenase expression and prostaglandin production in a mouse model of allergic airway inflammation. *Immunol. Lett.* 106, 72–81.
- Gali-Muhtasib, H., Roessner, A., Schneider-Stock, R., 2006. Thymoquinone: a promising anti-cancer drug from natural sources. *Int. J. Biochem. Cell Biol.* 38, 1249–1253.
- Gholamnezhad, Z., Rafatpanah, H., Sadeghnia, H.R., Boskabady, M.H., 2015a. Immunomodulatory and cytotoxic effects of *Nigella sativa* and thymoquinone on rat splenocytes. *Food Chem. Toxicol.* 86, 72–80.
- Gholamnezhad, Z., Keyhanmanesh, R., Boskabady, M.H., 2015b. Anti-inflammatory, antioxidant, and immunomodulatory aspects of *Nigella sativa* for its preventive and bronchodilatory effects of obstructive respiratory diseases: a review of basic and clinical evidence. *J. Funct. Foods* 17, 910–927.
- Gholamnezhad, Z., Havakhah, S., Boskabady, M.H., 2016. Preclinical and clinical effects of *Nigella sativa* and its constituents, thymoquinone: a review. *J. Ethnopharmacol.* 190, 372–386.
- Goreja, W.G., 2003. *Black Seed: Nature's Miracle Remedy*. Amazing Herbs Press, New York, NY, pp. 1–54.
- Greenbaum, D., Colangelo, C., Williams, K., Gerstein, M., 2003. Comparing protein abundance and mRNA expression levels on a genomic scale. *Gen. Biol.* 4, 117. <https://doi.org/10.1186/gb-2003-4-9-117>.
- Grewe, M., Bruijnzeel-Koomen, C.A., Schopf, E., Thepen, T., Langeveld-Wildschut, A.G., Ruzicka, T., Krutmann, J., 1998. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol. Today* 19, 359–361.
- Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G.G., Liu, Y., Chen, X., Li, L., Wu, S., Chen, Y., Jiang, H., Tan, L., Xie, J., Zhu, X., Liang, S., Deng, H., 2008. How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. *Acta Biochim. Biophys. Sin. (Shanghai)* 40 (5), 426–436.
- Hajhashemi, V., Ghannadi, A., Jafarabadi, H., 2004. Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug. *Phytother. Res.* 18, 195–199.
- Harris, D.P., Goodrich, S., Gerth, A.J., Peng, S.L., Lund, F.E., 2005. Regulation of INF- γ production by B effector 1 cells: essential roles for T-bet and the INF- γ receptor. *J. Immunol.* 174, 6781–6790.
- Hirota, Y., Tomokazu, I., Hideo, K., Shusei, A., Hiroyuki, T., Hiroichi, N., Naoki, I., 2010. Comparison of the efficacy of tacrolimus and cyclosporine a in a murine model of dinitrofluorobenzene-induced atopic dermatitis. *Eur. J. Pharmacol.* 645, 171–176.
- Homey, B., Steinhoff, M., Ruzicka, T., Leung, D.Y., 2006. Cytokines and chemokines orchestrate atopic skin inflammation. *J. Allergy Clin. Immunol.* 118, 178–189.
- Horikawa, T., Nakayama, T., Hikita, I., Yamada, H., Fujisawa, R., Bito, T., Harada, S., Fukunaga, A., Chantry, D., Gray, P.W., 2002. IFN- γ -inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epidermal keratinocytes and their roles in atopic dermatitis. *Int. Immunol.* 14 (7), 767–773.
- Horsmanheimo, L., Harvima, I.T., Jarvikallio, A., Harvima, R.J., Naukkarinen, A., Horsmanheimo, M., 1994. Mast cells are one major source of interleukin-4 in atopic dermatitis. *Br. J. Dermatol.* 131, 348–353.
- Hossen, M.J., Yang, W.S., Kim, D., Aravinthan, A., Kim, J.-H., Cho, J.Y., 2017. Thymoquinone: an IRAK1 inhibitor with in vivo and in vitro anti-inflammatory activities. *Sci. Rep.* 7, 42995. <https://doi.org/10.1038/srep42995>.
- Hwang, J.S., Kim, J.E., Yu, Y.B., Im, S.H., 2013. Modulation of experimental atopic dermatitis by topical application of Gami-Cheongyeul-Sodok-Eum. *BMC Complement. Altern. Med.* 13, 312. [online] Available at: <http://www.biomedcentral.com/1472-6882/13/312/> [Accessed 12 April 2017].
- Jin, H., He, R., Oyoshi, M., Geha, R.S., 2009. Animal models of atopic dermatitis. *J. Invest. Dermatol.* 129 (1), 31–40.
- Kang, J.S., Yoon, W.K., Han, M.H., Lee, H., Lee, C.W., Lee, K.H., Han, S.B., Lee, K., Yang, K.H., Park, S.K., Kim, H.M., 2008. Inhibition of atopic dermatitis by topical application of silymarin in NC/Nga mice. *Int. Immunopharmacol.* 8, 1475–1480.
- Keyhanmanesh, R., Boskabady, M.H., Eslamizadeh, M.J., Khamneh, S., Ebrahimi, M.A., 2010a. The effect of thymoquinone, the main constituent of *Nigella sativa* on tracheal responsiveness and white blood cell count in lung lavage of sensitized guinea pigs.

- Planta Med. 76 (3), 218–222.
- Keyhanmanesh, R., Boskabady, M.H., Khamneh, S., Doostar, Y., 2010b. Effect of thymoquinone on the lung pathology and cytokine levels of ovalbumin-sensitized guinea pigs. *Pharmacol. Rep.* 62 (5), 910–916.
- Kim, S.R., Choi, H.S., Seo, H.S., Choi, Y.K., Shin, Y.C., Ko, S.G., 2012. Topical application of herbal mixture extract inhibits ovalbumin- or 2,4-dinitrochlorobenzene-induced atopic dermatitis. *Evid. Complement. Alternat. Med.* 2012 [online] Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23304205> [Accessed 2 January 2017].
- Kim, H., Kim, J.R., Kang, H., Choi, J., Yang, H., Lee, P., Kim, J., Lee, K.W., 2014. 7,8,4'-trihydroxyisoflavone attenuates DNCB-induced atopic dermatitis-like symptoms in NC/Nga mice. *PLoS One* 9 (8), 1–9.
- Kondo, S., Yazawa, H., Jimbow, K., 2001. Reduction of serum interleukin-5 levels reflect clinical improvement in patients with atopic dermatitis. *J. Dermatol.* 28, 237–243.
- Krakowski, A.C., Eichenfield, L.F., Dohil, M.A., 2008. Management of atopic dermatitis in the pediatric population. *Pediatrics* 122 (4), 812–824.
- Kundu, J.K., Liu, L., Shin, J.W., Surh, Y.J., 2013. Thymoquinone inhibits phorbol ester-induced activation of NF- κ B and expression of COX-2, and induces expression of cytoprotective enzymes in mouse skin in vivo. *Biochem. Biophys. Res. Commun.* 438, 721–727.
- Lee, K.L., Jeong, E.S., Heo, S.H., Seo, J.H., Jeong, D.G., Choi, Y.K., 2010. A novel model for human atopic dermatitis: application of repeated DNCB patch in BALB/c mice, in comparison with NC/Nga mice. *Lab. Anim. Res.* 26 (1), 95–102.
- Leung, D.Y., Bieber, T., 2003. Atopic dermatitis. *Lancet* 361, 151–160.
- Li, Y.Z., Lu, X.Y., Jiang, W., Li, L.F., 2013. Anti-inflammatory effect of qingpeng ointment in atopic dermatitis-like murine model. *Evid. Complement. Altern. Med.*, 907016. <https://doi.org/10.1155/2013/907016>. Epub 2013 Aug 21.
- Lim, H.S., Ha, H., Lee, H., Lee, J.K., Lee, M.Y., Shin, H.K., 2014. *Morus alba* L. suppresses the development of atopic dermatitis induced by the house dust mite in NC/Nga mice. *BMC Complement. Altern. Med.* 14, 1–9.
- Matsuoka, H., Maki, N., Yoshida, S., Arai, M., Wang, J., Oikawa, Y., Ikeda, T., Hirota, N., 2003. A mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. *Allergy* 58, 139–145.
- Meingassner, J.G., Fahrngruber, H., Bavandi, A., 2003. Pimecrolimus inhibits the elicitation phase but does not suppress the sensitization phase in murine contact hypersensitivity, in contrast to tacrolimus and cyclosporine A. *J. Invest. Dermatol.* 121, 77–80.
- Navi, D., Saegusa, J., Liu, F.T., 2007. Mast cells and immunological skin diseases. *Clin. Rev. Allergy Immunol.* 33 (1–2), 144–155.
- Novak, N., 2009. New insights into the mechanism and management of allergic diseases: atopic dermatitis. *Allergy* 64, 265–275.
- Novak, N., Bieber, T., Leung, D.Y., 2003. Immune mechanisms leading to atopic dermatitis. *J. Allergy Clin. Immunol.* 112, 128–139.
- Ofori, A.O., Corona, R., 2017. What's New in Dermatology. [online] Available at: [Accessed 3 March 2017]. <https://www.uptodate.com/contents/whats-new-in-dermatology#H112565>.
- Ong, P.Y., Leung, D.Y., 2006. Immune dysregulation in atopic dermatitis. *Curr. Allergy Asthma Rep.* 6, 384–389.
- Pillars, P., 2006. Immunosuppressive mechanisms of action and monitoring. *Aust. Prescr.* 29, 99–101.
- Romagnani, S., 2000. The role of lymphocytes in allergic disease. *J. Allergy Clin. Immunol.* 105, 399–408.
- Rothenberg, M.E., Hogan, S.P., 2006. The eosinophil. *Annu. Rev. Immunol.* 24, 147–174.
- Shabbir, A., Shahzad, M., Ali, A., Zia-ur-Rehman, M., 2014. Anti-arthritis activity of N-(2,4-dihydroxyphenyl) methylidene]-2-(3,4-dimethyl-5,5-dioxidopyrazolol[4,3-] [1,2]benzothiazin-1(4H)-yl) acetohydrazide. *Eur. J. Pharmacol.* (738), 263–272.
- Soter, N.A., 1989. Morphology of atopic eczema. *Allergy* 44 (9), 16–19.
- Spergel, J.M., Paller, A.S., 2003. Atopic dermatitis and the atopic march. *J. Allergy Clin. Immunol.* 112 (Suppl. 6), 118–127.
- Spergel, J.M., Mizoguchi, E., Oettgen, H., Bhan, A.K., Geha, R.S., 1999. Role of Th1 and Th2 cytokines in a murine model of allergic dermatitis. *J. Clin. Invest.* 103, 1103–1111.
- Tekeoglu, I., Dogan, A., Ediz, L., Budancamanak, M., Demirel, A., 2007. Effects of thymoquinone (Volatile Oil of Black Cumin) on rheumatoid arthritis in rat models. *Phytother. Res.* 21, 895–897.
- Thomson, A.W., Bonham, C.A., Zeevi, A., 1995. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. *Ther. Drug Monit.* 17 (6), 584–591.
- Trautmann, A., Akdis, M., Kleemann, D., Altnauer, F., Simon, H.-U., Graeve, T., Noll, M., Eva-B, B., Blaser, K., 2000. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J. Clin. Invest.* 106 (1), 25–35.
- Werfel, T., Morita, A., Grewe, M., Renz, H., Wahn, U., Krutmann, J., Kapp, A., 1996. Allergen specificity of skin-infiltrating T cells is not restricted to type-2 cytokine pattern in chronic skin lesions of atopic dermatitis. *J. Invest. Dermatol.* 107, 871–876.
- Wu, C.T., Huang, K.S., Yang, C.H., Chen, Y.C., Liao, J.W., Kuo, C.L., Chen, C.L., Lo, S.F., Hsieh, C.C., Tsay, H.S., 2014. Inhibitory effects of cultured *Dendrobium tosaense* on atopic dermatitis murine model. *Int. J. Pharm.* 463, 193–200.
- Yamamoto, T., Nishioka, K., 2003. Topical tacrolimus: an effective therapy for facial psoriasis. *Eur. J. Dermatol.* 13, 471–473.
- Yoshimoto, T., Mizutani, H., Tsutsui, H., Noben-Trauth, N., Yamanaka, K., Tanaka, M., Izumi, S., Okamura, H., Paul, W.E., Nakanishi, K., 2000. IL-18 induction of IgE: dependence on CD4+ T cells, IL-4 and STAT6. *Nat. Immunol.* 1 (2), 132–137.
- Yurttaş, V., Şerefican, M., Erkoçoğlu, M., Dagli, M., Terzi, E.H., Fırat, T., Seyhan, S., 2016. Comparison of histopathological effects of thymoquinone and local nasal corticosteroids in allergic rhinitis in a rabbit model. *ORL* 78 (1), 55–60.
- Zhou, S.L., Tan, G.H., Huang, F.Y., Wang, H., Lin, Y.Y., Chen, S.L., 2014. Sanpao herbs inhibit development of atopic dermatitis in Balb/c mice, 2018 inhibit development of atopic dermatitis in Balb/c mice. *Asian Pac. J. Allergy Immunol.* 32, 140–144.