



# Curcumin Ameliorates Ovalbumin-Induced Atopic Dermatitis and Blocks the Progression of Atopic March in Mice

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**Abstract**—Curcumin, extracted from the roots of *Curcuma longa*, has been used as an anti-inflammatory agent since the time of Ayurveda. The present work was designed to evaluate the potential of curcumin in amelioration of ovalbumin (OVA) induced AD in mice. Female BALB/c mice were subjected to skin OVA-patch application for a period of 1 week followed by resting period of 2 weeks, and the same protocol was repeated thrice. Curcumin was administered daily at dose of 20 mg/kg (i.p.) for 7 consecutive days during last sensitization phase. The phytochemical ameliorated the OVA-induced skin pathology as evident by normalization of epidermal thickness and suppressed infiltration of inflammatory cells in dermal region. The expression of Th2 promoting cytokines (TSLP/IL-33) and Th2 cytokines (IL-4/IL-5/IL-13/IL-31) was suppressed markedly along with reduced STAT-6 phosphorylation and GATA-3 expression. Curcumin administration also restored the redox balance and phosphorylation status of P65-NF-κB. Additionally, the epicutaneously sensitized mice challenged with aerosolized OVA developed asthmatic features which were effectively thwarted back upon curcumin treatment as reflected by data on total/differential cells in BALF and mRNA expression of Th2 cytokines in lungs. Overall, our findings demonstrate that curcumin treatment blunts the development of AD as well as associated atopic march in experimental mice.

**KEY WORDS:** atopic dermatitis; curcumin; atopic march; asthma; ovalbumin.

## INTRODUCTION

Atopic dermatitis (AD) is a Th2-dominant skin inflammatory disorder which majorly affects children (10–20 %) [1, 2]. It is a result of skin barrier disruption and dysregulated

immune system [3]. Allergen exposure to the impaired skin induces itching and scratching, resulting into the formation of eczematous skin lesions which are the hallmarks of AD. It further leads to the production of epithelial cell-derived cytokines, mainly, thymic stromal lymphopoietin (TSLP) and IL-33 [4, 5]. TSLP activates the skin laden dendritic cells, which further stimulates the differentiation and proliferation of T cells toward Th2 phenotype [6, 7]. IL-33 is another cytokine which is known to promote the expression of Th2 cytokines such as IL-4, IL-5, IL-13, and IL-31 [8]. Overall, acute AD lesions display infiltration of inflammatory cells in the dermal regions as well as thickening of epidermal region along with increased expression of Th2

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cytokines [9, 10]. The accumulation of inflammatory cells results in oxidative stress conditions, which further exacerbates the inflammatory response in the skin [4].

The early onset of AD often leads to the sequential development of asthma and allergic rhinitis; which is termed as atopic march [1, 2, 5, 11, 12]. Indeed, reports suggest that 20 % of children with mild AD develop asthma and more than 60 % with severe AD develop asthma [13]. In fact, Spergel et al. have reported that epicutaneous sensitization with ovalbumin (OVA) followed by airway challenge induces asthmatic features such as airway inflammation and airway hyperresponsiveness in mice [14].

Till date, there is no treatment available which can completely ameliorate the symptoms of AD. However, currently available therapies target against Th2-mediated inflammatory responses but their long-term use is linked with several adverse effects such as skin atrophy, hyper- and hypo-pigmentation and skin burning [15, 16]. Therefore, a cure for AD with minimal side effects is needed. Also, there is a hypothesis that therapies targeting the amelioration of AD-like symptoms in infants may possibly prevent the subsequent advancement of the disease toward asthma [17].

Curcumin is a natural polyphenol, which is an active constituent of turmeric (*Curcuma longa*), and is well known for its anti-inflammatory properties [18, 19]. Moreover, the protective potential of curcumin has been reported in various allergic diseases including asthma [20, 21]. Also, the compound has been shown to possess beneficial effects against TNBC-induced skin swelling [22]. Further, Moon et al. have reported that TSLP (a critical player in AD and atopic march) expression was downregulated by curcumin in human mast cell line (HMC-1) [23]. In light of the facts stated above, the present work was designed to examine the potential beneficial effects of curcumin on manifestation of AD and associated atopic march using mouse model.

## MATERIAL AND METHODS

### Drugs and Chemicals

OVA and Curcumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals used in the study were of analytical grade. Other chemicals required were purchased from HiMedia Laboratories, Mumbai, India, and Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Antibodies used for western blot were purchased from Santa Cruz Biotechnology (Finnell St, Dallas, Texas) and R&D Systems (McKinley place, NE, Minneapolis).

### Animals

Six-week-old female BALB/c mice weighing 20–25 g were used for the experiments, which were procured from the central animal house facility, Panjab University, Chandigarh. All the animals were housed in polypropylene cages and were allowed unlimited access to sterilized chow and water. Animal care and all experimental procedures were approved and conducted in accordance with the “Guidelines for the Care and Use of Experimental Animals” as approved by Institutional Animal Ethical Committee (PU/IAEC/S/16/42). Animals were acclimatized to the laboratory conditions for 1 or 2 weeks prior to experimentation.

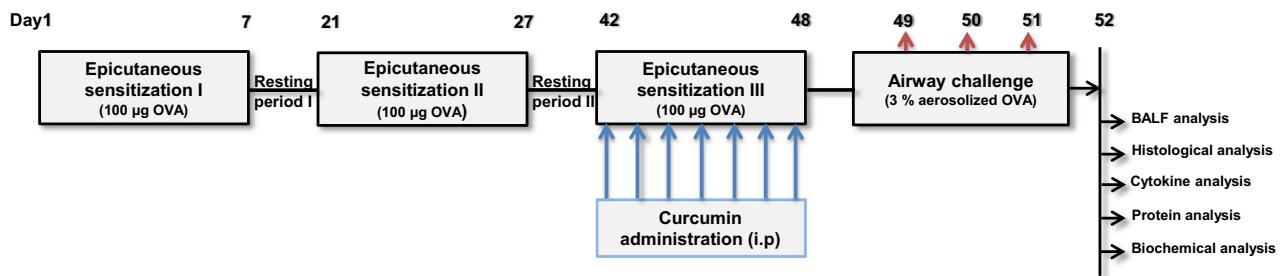
### Experimental Design

Mice were divided into four groups (5–6 animals in each group) as explained below:

- i. Control group: Mice were given standard diet. No allergen and drug was given to them.
- ii. Curcumin only (Cur) group: Curcumin was given to the mice at dose of 20 mg/kg, without any kind of allergen exposure.
- iii. OVA group: Mice were sensitized and challenged with OVA as per detail given in Fig. 1.
- iv. OVA + curcumin (OVA + Cur) group: OVA sensitized and challenged mice were administered curcumin by intraperitoneal injection. Curcumin was administered once daily at different doses of 10, 20, and 30 mg/kg body weight, for a total period of 7 days during last sensitization phase.

### Sensitization and Challenge

Animals were sensitized epicutaneously using OVA as allergen according to Fig. 1. Briefly, the back skin hair of the mice were shaved with an electric razor and then the skin was tape stripped (6X). On a patch of sterile gauze, 100 µg of OVA was applied. The patch was placed on the skin for a period of 1 week and then removed. After 2 weeks, the patch was placed again at the same skin site and the process was repeated twice. Overall, each mouse had an exposure to OVA for a total period of 3 weeks with a couple of recovery periods as depicted in Fig. 1 [14]. Curcumin at different doses (10, 20, and 30 mg/kg b.wt.) was administered intraperitoneally daily during last week of sensitization (Fig. 1). Twenty-four hours after the last curcumin treatment, the animals were euthanized by cervical dislocation.



**Fig. 1.** Sensitization and challenge protocol. Mice were sensitized with a patch of OVA (100 µg), applied on the shaved back skin. The patch was placed for a period of 1 week and then removed. After 2 weeks, an identical patch was reapplied to the same skin site. Each mouse had a total of 3-week exposures to OVA separated by a 2 week intervals. Curcumin was administered during the last sensitization phase. On days 48, 49, and 50, the mice were challenged with aerosolized OVA (3 %). All experiments were done after 24 h of the last sensitization/challenge

For examining the impact of curcumin on OVA-mediated atopic march, a group of animals were challenged with aerosolized OVA (3 %) for 3 consecutive days after the last sensitization phase. Mice were placed in plexiglass chamber for 30 min, which is connected to a nebulizer that generates aerosolized OVA [24]. The animals were sacrificed 24 h after the last OVA exposure for procurement of bronchoalveolar lavage fluid (BALF) and lung tissue.

### Curcumin Treatment

Curcumin (Sigma, St. Louis, MO, USA) was administered by intraperitoneal injection at a dose of 20 mg/kg body weight once daily for 7 days during the last sensitization phase.

### BALF Analysis

The lungs were lavaged with 1 ml of phosphate buffer saline (0.1 M PBS, pH 7.4). BALF samples were centrifuged at 1,600 rpm for 8 min at 4 °C to obtain cell pellet. The pellet was re-suspended in PBS and total cell count was done using hemocytometer. Further, a cell smear was prepared on microscopic slides using cytospin centrifuge, which were stained with hematoxylin and eosin (H&E) for differential cell count assessment [25].

### Histological Examination

Skin specimens were fixed in 10% formalin (prepared in 0.1 M PBS) and slides of 5 µm thin sections were prepared. The sections were then stained with H&E to examine the epidermal thickness and infiltration of inflammatory cells in the dermal area. The images of the stained sections were obtained at 100 X and 400 X [14]. Epidermal and dermal thickness (in µm) was analyzed in H&E stained sections viewed under magnification of 100 X and 400 X,

respectively, using Image-Pro 10 software. Thickness was measured in five randomly selected fields from each sample.

### RNA Isolation and Conventional Reverse Transcriptase PCR

Total RNA was isolated from skin or lung tissues of different groups using TRIzol™ reagents as per the manufacturer's instructions. cDNA synthesis was carried out using iScript cDNA synthesis kit (BioRad) as per the manufacturer's protocol. The expression values of each target gene were normalized to the expression values of Actin. The analyzed target genes were IL-4, IL-5, IL-13, IL-31, and IL-33 (sequences of these primers are given in Table 1). The amplification program involved 35 cycles of: pre-incubation of 5 min at 95 °C and then amplification cycles consisting of 30 s at 95 °C, 45 s at 60 °C, and 1 min at 72 °C. The PCR products were then incubated for 15 min at 72 °C. The resulting PCR products were analyzed by agarose gel electrophoresis. The intensity of the band area in electrophoresed gels was measured in ImageJ software (NIH, Bethesda, MD, USA) [24].

### Western Blot

Skin tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitor. The lysates were centrifuged at 6,000 rpm for 10 min at 4 °C. Total protein content was estimated in the supernatant by using Lowry's method. Samples were run on 10% SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was blocked with 3% BSA for 1 h at room temperature and then incubated overnight with primary antibodies against phospho-P65-NF-κB (Ser 536) (1:1000), total NF-κB (1:1000), phospho-STAT-6 (1:1000), total STAT-6 (1:1000), GATA-3 (1:1000), TSLP (1:5000), and Actin (1:2000) at 4 °C as reported previously [26]. After the

**Table 1.** Primer sequences

Gene	NCBI ID	Forward primer (5'-3')	Reverse primer (5'-3')
IL-4	NM_021283.2	TCAACCCCCAGCTAGTTGTC	TGTTCTTCGTTGCTGTGAAG
IL-5	NM_010558.1	ATGGAGATTCCCATGAGCAC	GTCCTCTCCTGCCACACTTC
IL-13	NM_008355.3	CAGCTCCCTGGTTCTCTCAC	CCACACTCCATACCATGCTG
IL-31	NM_029594.1	TCGGTCATCATAGCACATCTGGA	GCACAGTCCCTTGAGTTAAGT
IL-33	NM_001164724.2	CAGGCCTTCTCGTCCTTCAC	TCTCTCCACTAGAGGCCAGCTG
$\beta$ -Actin	NM_007393.5	TACAGCTTCACCACACCACAGC	TCTCCAGGGAGGAAGAGGAT

incubation of primary antibodies, the membrane was washed with 1X PBST (0.05% Tween-20), thrice at RT. This was followed by the incubation with horseradish peroxidase labelled-secondary antibodies at a dilution of 1:10,000 in PBS for 1 h at RT. Protein signals were detected via chemiluminescence using Clarity™ western blotting substrate. ImageJ software was used for the densitometric analysis (NIH, Bethesda, MD, USA).

### Biochemical Analysis

Different biochemical assays were performed in freshly procured skin tissues. The whole tissue homogenates were prepared in ice-cold PBS (pH 7.4) at 4 °C, and the supernatants were collected following centrifugation at 6,000 rpm for 10 min at 4 °C. The total protein content was measured according to the Lowry et al. [27]. Reactive oxygen species (ROS) levels were measured using DCFDA dye. Tissue homogenates were mixed with DCFDA (10 µM) and succinate (0.1 mM) and incubated at 37 °C for 1 h. The fluorescence was measured (485 nm emission and 520 nm exCitation) using Shimadzu RF5301, Kyoto, Japan [28]. Malondialdehyde (MDA) levels as an indicator of lipid peroxidation were quantified. Samples were incubated with Tris-Cl buffer, pH 7.4 (0.1 M) at 37 °C for 2 h. Then, 10 % of ice-cold TCA was added to stop the reaction and centrifuged at 2,000 rpm for 10 min. 0.67 % TBA was mixed with the supernatants and boiled the mixtures at 100 °C for 10 min. Absorbance was measured at 532 nm using Shimadzu UV-1800 spectrophotometer [29]. Oxidative protein damage was determined by measuring the formation of DNP which is a measure of protein carbonyl moieties. Homogenates were mixed with DNPH (20 mM prepared in 2 M HCl) and simultaneously a negative control for each sample was used, in which the samples were mixed with HCl. The tubes were incubated in the dark for 1 h with vortexing after every 15 mins. TCA was added to each tube and the suspensions were centrifuged at 10,000 g for 10 min at 4 °C. The pellets were resuspended in 1 ml of ethanol/ethyl acetate (1:1) and again

centrifuged at 10,000 g for 10 mins at 4 °C. Finally, the pellet was resuspended in 500 µl of G-HCl and mixed till the appearance of yellow color. The intensity of the yellow color was measured against the negative controls at 370 nm using Shimadzu UV-1800 spectrophotometer [30]. Superoxide dismutase levels were determined using method described by Kono, 1978 [31]. Samples were mixed with solution A [50 mM sodium carbonate and 0.1 mM EDTA, pH 10.4], solution B [96 mM Nitroblue tetrazolium], solution C [0.6 % (w/v) Triton X-100], and solution D [20 mM hydroxylamine hydrochloride] and the blue color was observed. Then, the absorbance was measured at 560 nm for 3 min at an interval of 30 s using Shimadzu UV-1800 spectrophotometer. Catalase levels were measured by adding 3 ml of H<sub>2</sub>O<sub>2</sub> [12.5 mM H<sub>2</sub>O<sub>2</sub> in 0.067 M sodium phosphate buffer, pH 7.0] to the sample. The decrease in absorbance was measured at 240 nm for 3 min [32]. Reduced glutathione (GSH) content was analyzed according to the Moron et al. (1979) methods. Samples were incubated with 4 % sulphosalicylic acid and centrifuged at 5,000 rpm for 10 min. Supernatants were mixed with Ellman's reagent (0.1 mM DTNB, prepared in 0.1 M sodium phosphate buffer). Absorbance was measured at 412 nm using Shimadzu UV-1800 spectrophotometer [33].

### Statistical Analysis

Data were expressed as the mean  $\pm$  standard error of the mean. Statistical analysis was done by one-way analysis of variance (ANOVA) tests followed by Tukey's multiple comparisons using graph-pad prism software (GraphPad Software, Inc. La Jolla, CA).

## RESULTS

### Curcumin Ameliorates OVA-Induced AD-Like Phenotypic and Histologic Changes in Mice

AD skin lesions are characterized by increased skin thickness and dermal infiltration of inflammatory cells

[34]. Thus, first we examined the development of AD-like features and found out that repeated application of OVA to skin induced the development of skin rashes in mice, as expected. However, administration of curcumin at different doses, i.e., 10, 20, and 30 mg/kg body weight, daily during last sensitization phase ameliorated the skin rashes remarkably, particularly at a dose regimen of 20 and 30 mg/kg dose (Fig. 2a). Next, histological examination was conducted using skin tissues derived from mice given different doses of curcumin (Fig. 2b and Online Resource 1). Data clearly showed that the phytochemical at the dose of 20 and 30 mg/kg restores histo-architecture of skin markedly as reflected by the normalization of epidermal thickness (Fig. 2c) and recruitment of inflammatory cells in dermal regions. As dose of 20 and 30 mg/kg seems to work equally well, we performed the rest of the experiments with the minimal effective dose, i.e., 20 mg/kg.

### Curcumin Downregulates the Enhanced Expression of Th2 Promoting Cytokines Upon Epicutaneous OVA Application

As stated earlier, epithelial cell-derived cytokines such as TSLP and IL-33 play a critical role in the initiation of the allergic process [35, 36]. We first examined their expression in the skin samples derived from mice given various treatments. Data shown in Fig. 3a–b clearly indicates that epicutaneous OVA sensitization resulted in 1.5 fold ( $P < 0.01$ ) increase in protein levels of TSLP in the skin tissues of AD mice when compared with the control mice. Interestingly, curcumin administration in OVA-induced AD mice down regulates the expression of the cytokine, significantly (60 %,  $P < 0.001$ ) (Fig. 3b). Furthermore, our data (Fig. 3c) shows that curcumin treatment also results in downregulation of mRNA expression of IL-33 (98 %,  $P < 0.01$ ).

### Curcumin Suppresses the Expression of Th2 Cytokines Potentially by Down-Regulating the Activation/Expression of STAT-6 and GATA-3

Given the fact that AD is strongly associated with the enhanced expression of Th2 cytokines, we also examined the expression of these cytokines in skin tissues using conventional PCR. Figure 4a–b showed that the expression of Th2 cell-cytokines, viz., IL-4, IL-5, IL-13, and IL-31 was elevated significantly (4.9-fold,  $P < 0.01$ ; 4.8-fold,  $P < 0.001$ ; 3.4-fold,  $P < 0.01$ ; and 10-fold,  $P < 0.001$ , respectively) in skin tissue of OVA sensitized mice; whereas, curcumin treatment was able to blunt the expression of these cytokines, significantly (65 %,  $P < 0.01$ ; 53 %,  $P < 0.001$ ; 47 %,  $P < 0.01$ ; and 84 %,  $P < 0.001$ , respectively).

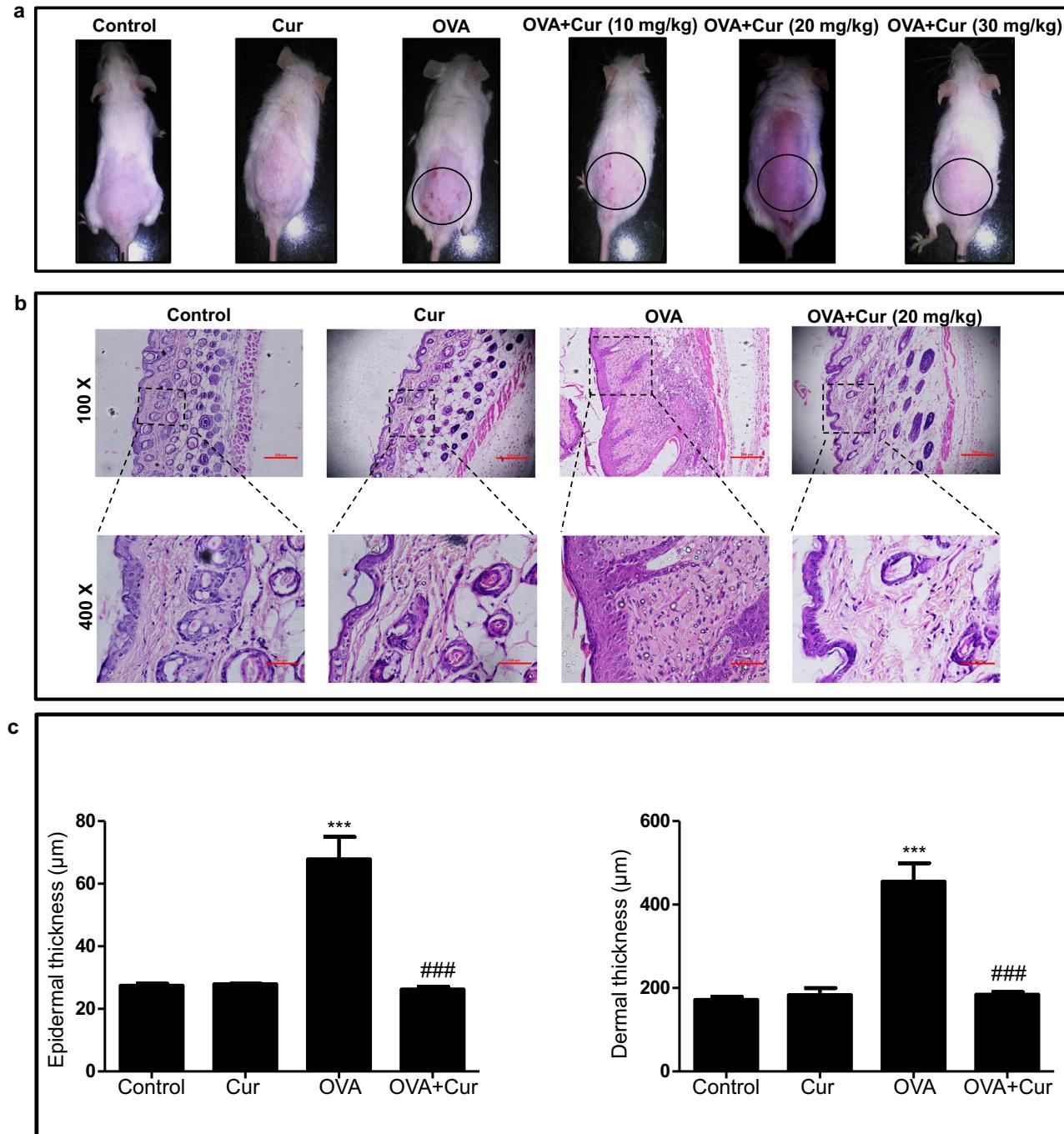
In order to gain insight into the molecular mechanism behind the expression of these cytokines, we investigated the activation and expression of phospho-STAT-6 (Tyr-641) and GATA-3, respectively, which are master regulators of Th2 cell differentiation. Our data confirm that curcumin downregulates the phosphorylation of STAT-6 (59.9%,  $P < 0.001$ ). Further, reduced phosphorylation of STAT-6 was accompanied by subdued expression of GATA-3 (56.3 %,  $P < 0.001$ ) in skin tissue of mice subjected to OVA sensitization (Fig. 4f–i).

### Curcumin Administration Restores the OVA-Induced Redox Imbalance

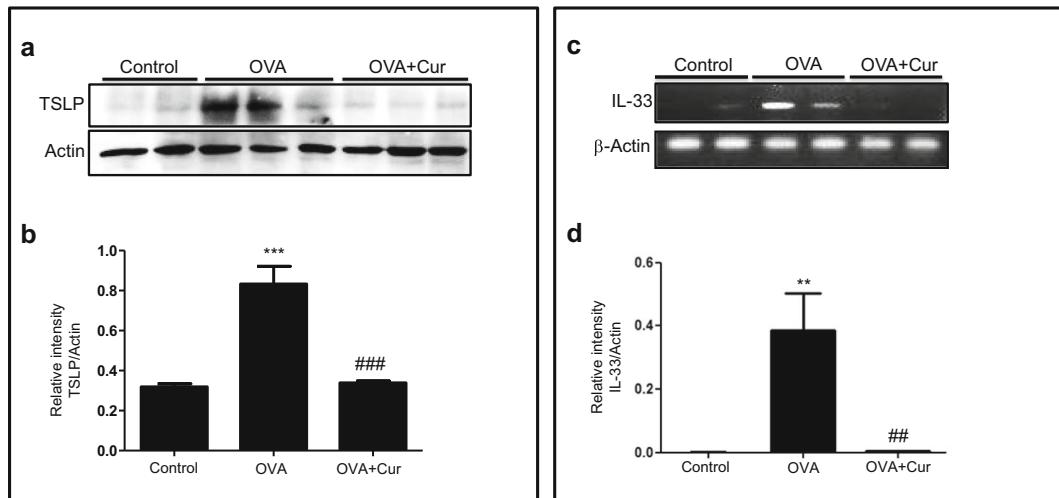
It is well known that inflammatory cells release various reactive oxygen intermediates and cause oxidative damage which further leads to the propagation of skin inflammation [37]. It is well known that curcumin tends to alter the imbalance between oxidant and antioxidant species in various inflammatory diseases [38]. Thus, we quantified the levels of ROS in skin tissues. Also, the levels of MDA and DNP were measured, which are indicative of ROS-mediated lipid peroxidation and protein damage, respectively. Our data show that OVA sensitization increased the ROS (84 %,  $P < 0.001$ ), MDA (99 %,  $P < 0.01$ ), and DNP levels (75.2 %,  $P < 0.001$ ) w.r.t control group. However, curcumin treatment restored back the levels of ROS (59.1 %,  $P < 0.001$ ), MDA (86.2 %,  $P < 0.01$ ), and DNP (49.8 %,  $P < 0.05$ ) toward normal, when compared with the OVA group. Further, reduction in OVA-induced oxidative damage by curcumin was associated with restoration of reduced glutathione content (71.2 %,  $P < 0.05$ ) and activities of SOD (64.2 %,  $P < 0.001$ ) and catalase (55 %,  $P < 0.01$ ) in skin tissue (Fig. 5a–f). Since, the interaction between ROS and NF- $\kappa$ B signaling pathway has already been reported; we examined the effect of curcumin on the activation of NF- $\kappa$ B by analyzing the phosphorylation of P65 subunit of NF- $\kappa$ B at ser 536 residue. Figure 5 g–h shows that curcumin suppresses the OVA-induced phosphorylation of P65-NF- $\kappa$ B, significantly (60.8 %,  $P < 0.001$ ).

### Curcumin Suppresses the Airway Inflammation via Downregulating the Expression of Th2 Cytokines in the Lung Tissues

Finally, we examined whether curcumin may modulate the development of allergic airway inflammation upon aerosolized exposure of OVA into the lungs of AD mice. Accordingly, epicutaneously sensitized AD mice were challenged with OVA for 3 consecutive days (as directed



**Fig. 2.** Curcumin ameliorates OVA-induced AD-like phenotypic and histologic changes in mice. (a) Curcumin treatment prevented the development of skin rashes in mice upon OVA exposure. (b) Representative images of H&E stained skin sections of saline sensitized, curcumin only, OVA-sensitized and curcumin-treated AD mice; Magnification 100 X and 400 X. (c) Comparison of the epidermal and dermal thickness in mice subjected to different treatments. Data shows mean  $\pm$  SEM, where  $n = 5-6$  mice per group (\*\* represents the significance between control and OVA-sensitized skin samples,  $P < 0.001$ ; # represents the significance between OVA and curcumin treated group,  $P < 0.001$ ). Cur, Curcumin; H&E, Hematoxylin, and Eosin; OVA, Ovalbumin



**Fig. 3.** Curcumin downregulates the enhanced expression of Th2 promoting cytokines upon epicutaneous OVA application. (a) Skin tissues of different groups were processed for western blot using antibody against TSLP and  $\beta$ -actin. (c) cDNA samples were amplified using conventional PCR to analyze the gene expression of IL-33. (b, d) Densitometric analysis was done to quantify the relative intensity of band area of TSLP and IL-33 against Actin. Data shows mean  $\pm$  SEM, where  $n = 5$ –6 mice per group (\*\*, \*\*\* represents the significance between control and OVA-sensitized skin samples,  $P < 0.01$  and  $P < 0.001$ , respectively; ##, ### represents the significance between OVA and curcumin treated group,  $P < 0.01$  and  $P < 0.001$ , respectively). Cur, Curcumin; OVA, Ovalbumin; TSLP, Thymic stromal lymphopoietin

in Fig. 1), and subsequently analysis of infiltration of inflammatory cells and expression of Th2 cytokines in lung tissues were carried out. BALF analysis shows 3.7-fold increase in the number of total inflammatory cells ( $P < 0.001$ ), 16-fold increase in lymphocytes number ( $P < 0.001$ ), and a drastic increase in the number of eosinophils ( $P < 0.001$ ) as compared to control group. However, curcumin administration in AD mice followed by challenge with OVA blocks the infiltration of inflammatory cells in airway as reflected by the number of total cells (62%,  $P < 0.001$ ), eosinophils (97 %,  $P < 0.001$ ), and lymphocytes (97 %,  $P < 0.001$ ) in BALF (Fig. 6a–c).

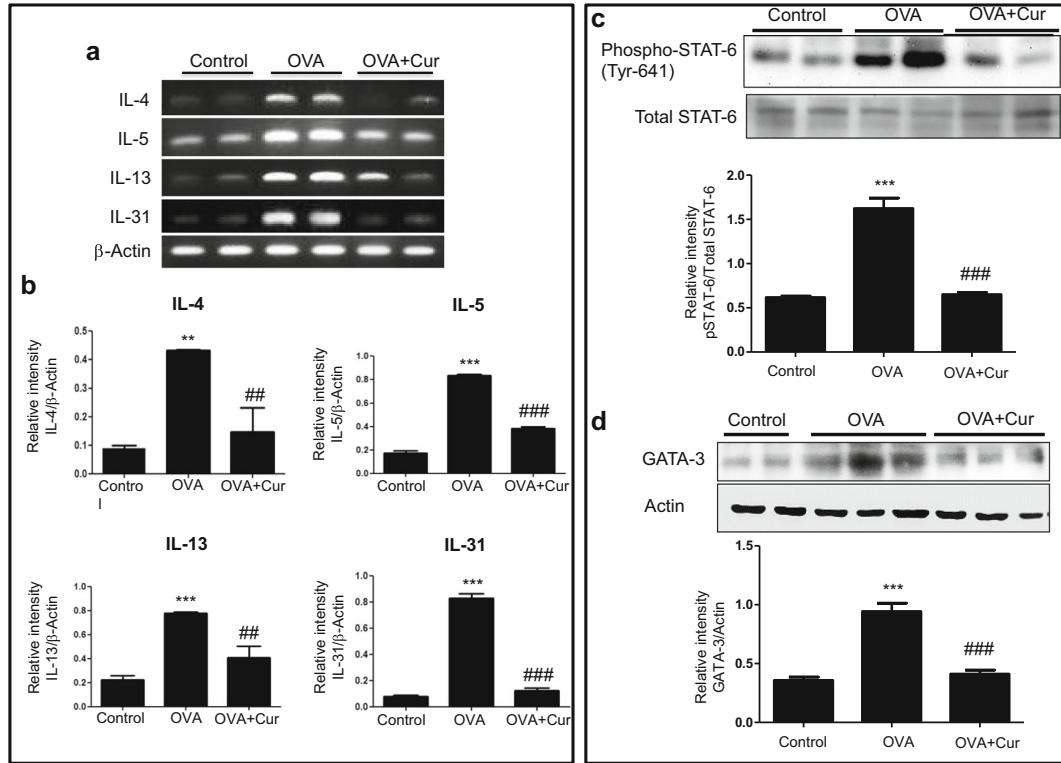
Next, the mRNA expression of IL-4, IL-5, and IL-13 in lung tissue was found to be decreased significantly in mice pre-treated with curcumin upon OVA challenge (73 %,  $P < 0.001$ ; 60 %,  $P < 0.001$ ; and 82 %,  $P < 0.001$ , respectively) (Fig. 6d–g). The reduction in the number of inflammatory cells in BALF of curcumin treated samples was found to be associated with the decreased production of inflammatory cytokines.

## DISCUSSION

AD is a chronic inflammatory condition of skin, characterized by intense itching and scratching leading to the formation of eczematous lesions [14, 39]. In majority of

the cases, the presence of AD during infancy increases the risk of developing other allergic diseases such as asthma and allergic rhinitis through atopic march [40]. This can be supported by the fact that once allergen-specific Th2 responses are generated, they can exert their effects systemically [4]. Therefore, therapies should be targeted against the initiation of AD which can prevent the development of asthma [41]. Taking into consideration, the numerous side effects of the currently available therapies targeting AD, a variety of natural compounds have proved to be safe. Curcumin has gained great attention due to its anti-inflammatory and antioxidant capacities in a number of disease conditions such as inflammatory bowel disease, cancer, neurodegenerative diseases and a variety of skin diseases including psoriasis, radiation wounds etc. [42]. In the present study, daily administration of curcumin for a total period of 7 days during last sensitization phase prevented the OVA-induced skin rashes and restored the underlying histological alterations. Protective potential of curcumin was further found to be associated with the reduced expression of Th2-promoting cytokines, Th2 cytokines, and the associated transcription factors. Moreover, curcumin was found to be effective in ameliorating asthmatic airway inflammation via downregulating the expression of Th2 cytokines in lung tissues.

Since AD involves the interplay between various cell types including T cells, langerhans cells, eosinophils, and

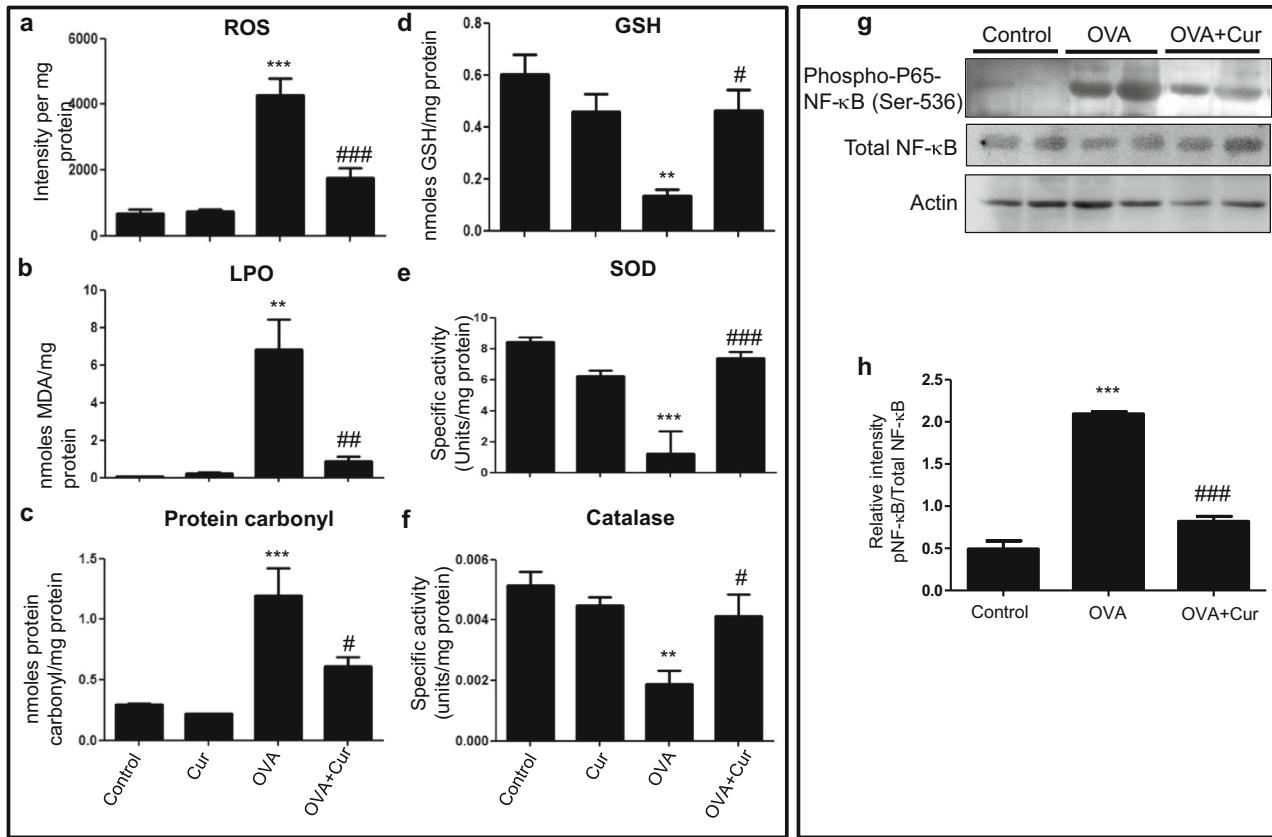


**Fig. 4.** Curcumin suppresses the expression of Th2 cytokines potentially by downregulating the expression of GATA-3 and STAT-6. (a) The expression of Th2 cytokines was analyzed by performing conventional PCR using primers for IL-4, IL-5, and IL-13 and IL-31. (b) Densitometric analysis was done to determine the relative intensity of the band area. (c, d) Activation/Expression of STAT-6 and GATA-3, Th2-related transcription factors were analyzed by western blot analysis using antibodies against Phospho-STAT-6 (Tyr 641), Total STAT-6, GATA-3, and  $\beta$ -ACTIN, and their relative band intensities were analyzed. Data represents mean  $\pm$  SEM, where  $n = 5$ –6 mice per group (\*\*, \*\*\* indicate the significance between control and OVA-sensitized skin samples,  $P < 0.01$  and  $P < 0.001$ , respectively; #, ### indicate the significance between OVA and curcumin-treated group,  $P < 0.01$  and  $P < 0.001$ , respectively). Cur, Curcumin; OVA, Ovalbumin

keratinocytes, keratinocytes play a seminal role in the pathogenesis of the disease due to their roles in skin barrier function and their contribution to the initiation and maintenance of inflammation [43]. Continuous allergen exposure to the skin impairs the barrier and stimulates the keratinocytes and other epithelial cells to release inflammatory mediators, such as TSLP and IL-33 [44]. TSLP has been reported as a critical player in the initiation of the allergic response [45]. To mimic the clinical/immunological features of human AD in mice, epicutaneous OVA exposure to the defective skin prompts the cells at barrier surfaces to release TSLP and IL-33. In agreement to this, our results also display elevated levels of these cytokines in OVA-induced AD skin while systemic curcumin administration led to the decrease in their levels. In fact, Moon et al. have reported that curcumin downregulates PMA-induced expression of TSLP in human mast cell line (HMC-1) [23]. Accordingly, it can be speculated that curcumin might interrupt the TSLP

signaling. Moreover, our data reveals that curcumin treatment produced clinical improvements in AD-like skin lesions such as reduction in the epidermal thickness and dermal infiltration of inflammatory cells. Also, the down-regulation in the expression of Th2 cytokines in curcumin treated mice can be linked with its inhibitory effect on GATA-3 and STAT-6 (Th2 associated transcription factors), which has been reported earlier in asthmatic conditions [46, 47]. Furthermore, there has been supporting evidence that TSLP activation mediates the generation of Th2 cell response [48, 49], hence it can be stated that curcumin interfere with the TSLP signaling cascade, thereby, suppressing the inflammatory responses in mice.

Furthermore, the infiltration of inflammatory cells in AD skin causes disturbance in redox balance that aggravates the condition by enhancing Th2 polarization [50]. It is well known that ROS production or oxidative stress hinders epidermal and dermal repair during inflammation [51]. Studies propose that oxidative injury to cellular lipid and protein

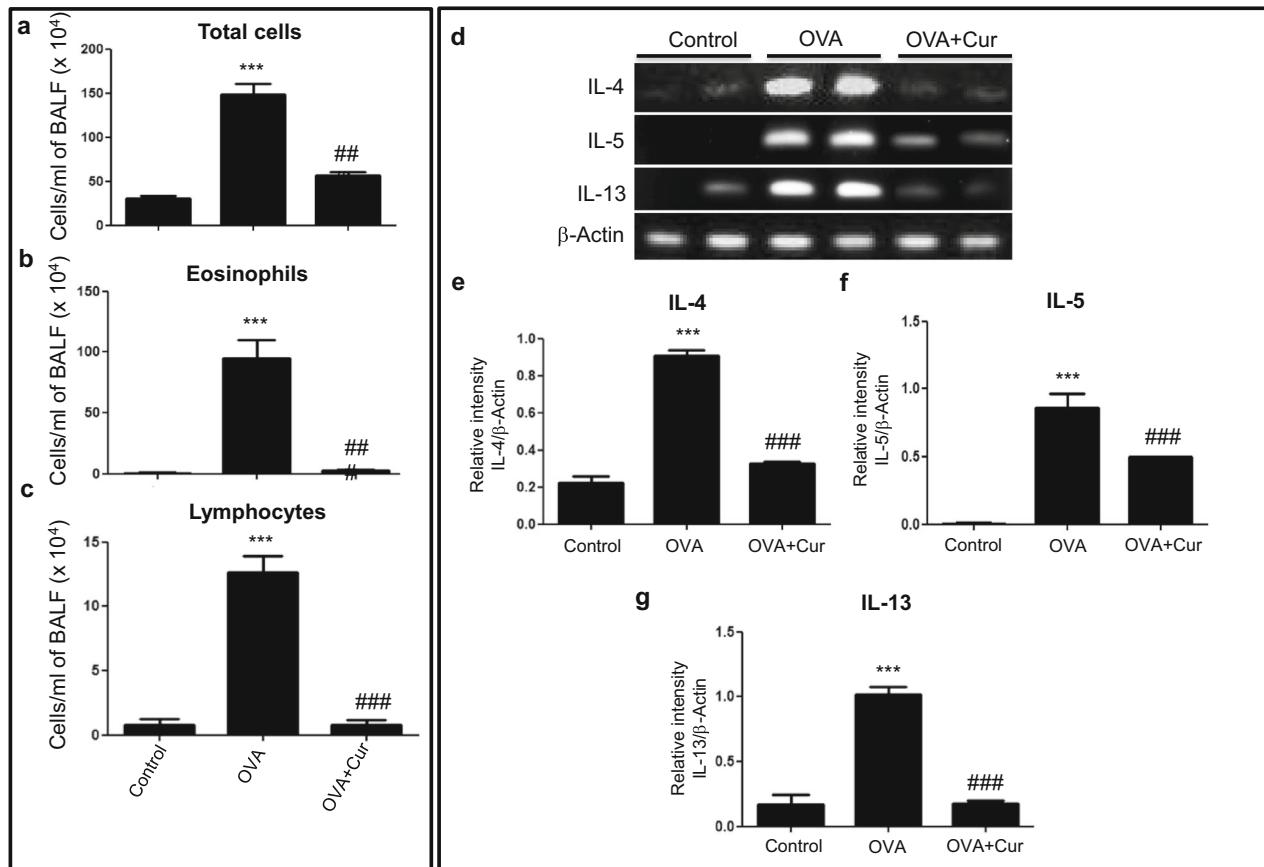


**Fig. 5. Curcumin administration restores the OVA-induced redox imbalance.** Skin tissue homogenates of different groups were processed to determine levels of markers of oxidative stress, (a) ROS (b) LPO (c) Protein carbonyls and antioxidant levels, (d) GSH (e) SOD and (f) catalase. Skin tissues of different groups were processed for western blot using antibody against phospho-P65-NF-κB, and total NF-κB (g, h) phosphorylation levels of P65 NF-κB (Ser 536) were analyzed using skin tissues of different groups by western blot analysis using antibodies against ph-P65-NF-κB and total NF-κB and relative band intensities were measured. The above data reflects mean  $\pm$  SEM, where  $n = 5-6$  mice per group (\*\*, \*\*\* represent the significance between control and OVA-sensitized skin samples,  $P < 0.01$  and  $P < 0.001$ , respectively; #, ##, ### represent the significance between OVA-sensitized and curcumin-treated group,  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively). Cur, Curcumin; LPO, Lipid peroxidation; OVA, Ovalbumin; ROS, Reactive oxygen species; SOD, Superoxide dismutase

content of cells plays a central role in pathogenesis of AD [52]. Our data on oxidative stress markers, ROS, MDA, and DNP along with counter players (GSH, SOD, and Catalase) clearly point toward the ability of curcumin to restore OVA-induced redox imbalance toward normal in skin tissue. Additionally, ROS drives the activation of NF-κB, which plays a central role in inflammation through regulation of the gene expression of various cytokines and other inflammatory mediators [53]. And, curcumin has been reported to affect many cellular processes involved in inflammation possibly via its free radical scavenging properties which further impact a number of downstream signaling molecules including NF-κB [54, 56]. Evidences suggest that curcumin prevents the activation of NF-κB [55]. Interestingly, our results also demonstrate the reduction in the levels of phosphorylation of NF-

κB. Therefore, the beneficial effects of curcumin observed by us can be linked with its antioxidant capacity and consequent suppression of NF-κB activation in addition to the suppression of TSLP release. Hence, it is possible that curcumin alleviates AD-like features potentially via direct modulation of the expression of TSLP and NF-κB.

Given the fact that half of the AD patients develop asthma at later stages of life, it appears that AD acts as an entry point for the sequential development of other allergic diseases. Animal studies confirm the development of asthmatic symptoms in epicutaneously sensitized mice upon OVA challenge [14, 57]. Our data corroborate the earlier findings that OVA induces the airway inflammation characterized by increased number of inflammatory cells in BALF and elevated expression of Th2 cytokines in the lungs as



**Fig. 6.** Curcumin suppresses the airway inflammation via downregulating the expression of Th2 cytokines in the lung tissues. Mice were epicutaneously sensitized and challenged with aerosolized OVA for 3 consecutive days after the last sensitization period. After 24 h from last challenge, mice were sacrificed and BALF analysis was performed. (a, b, c) BALF samples were used for counting of total cells, eosinophils, and lymphocytes. (d) The expression of Th2 cytokines was analyzed by performing conventional PCR using primers for IL-4, IL-5, and IL-13 in lung tissues subjected to different treatments (e, f, g). Densitometric analysis was done to determine the relative intensity of the band area. Data represents mean  $\pm$  SEM, where  $n = 5-6$  mice per group (\*\* indicate the significance between control and OVA-sensitized skin samples,  $P < 0.001$ ; ##, ### indicate the significance between OVA and Curcumin-treated group,  $P < 0.01$  and  $P < 0.001$ , respectively). Cur, Curcumin; OVA, Ovalbumin

compared to the control samples. Earlier, curcumin has been shown to exert its beneficial effect by alleviating airway inflammation when administered for a period of 21 days in OVA-induced asthmatic mice [21]. Indeed, our observations indicate that curcumin pre-treatment during the last epicutaneous sensitization period did not culminate into the asthmatic conditions upon subsequent OVA challenge. Therefore, this is the first study to show that amelioration of skin inflammation can impede the manifestation of asthma. Overall, it seems that curcumin pre-treatment can modulate the Th2 immune responses in lungs and thereby, halt the subsequent development of asthma manifestation.

In conclusion, our findings suggest that systemic curcumin administration can exert anti-inflammatory and

antioxidative effects in murine model of AD. In addition, suppression of AD-linked allergic airway inflammation upon exposure to aerosolized OVA provide evidence that curcumin could be a potential therapeutic agent for blunting the atopic march. Furthermore, it is proposed that curcumin based topical ointment may ameliorate not only AD symptoms but subsequent march toward asthmatic features. Indeed, effective use of topical curcumin has been reported in skin injuries and inflammation associated with rosacea, psoriasis, and photo-damaged skin but data on AD is lacking. Therefore, such topical curcumin-based ointments/creams which could be easily absorbed through the skin may be beneficial in

treatment of AD and prevention of associated allergic march.

## AUTHOR CONTRIBUTIONS

Conceptualization and design of experiments was done by Amarjit S Naura and Sukriti Sharma; Experiments were performed by Sukriti Sharma; Analysis of the data was done by Amarjit S Naura, Sukriti Sharma, and Gurupreet S Sethi. The draft of the manuscript was prepared by Sukriti Sharma and all authors reviewed and approved the final manuscript.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest.** The authors declare that they have no competing interests.

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