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ORIGINAL ARTICLE

Anti-inflammatory potential of hecogenin on atopic dermatitis and airway hyper-responsiveness by regulation of pro-inflammatory cytokines

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ABSTRACT

Objective: Hecogenin is a sapogenin found in Agave sisalana species that is used extensively for the treatment of anti-inflammatory, antifungal, hypotensive, anti-nociceptive activity and cancer. We have studied the anti-inflammatory effect of Hecogenin and its combination with Fluticasone on atopic dermatitis and airway hyper-responsiveness in Balb/c mice.

Material and methods: Dermatitis was induced by repeated application of 2, 4-dinitrofluorobenzene in Balb/c mice. After a topical application of Hecogenin, Fluticasone and their combination on the skin lesions, the ear thickness, ear weight and erythema score were evaluated. Asthma was induced by sensitization and challenge of ovalbumin in Balb/c mice.

Results: The topical application of Hecogenin and its combination with Fluticasone in mice effectively suppressed the ear swelling and weight. As well as the levels of pro-inflammatory cytokines were decreased by Hecogenin and its combination *in-vivo*. Whereas, intra-nasal administration of Hecogenin and its combination in ovalbumin induced airway hyper-responsiveness reveals a significant decrement in total cell count, differential cell count and cytokines levels. Similar observations were obtained for myeloperoxidase level in ear and lung tissue. The results were supported by histological studies of ear and lung tissue.

Conclusion: These data indicate that Hecogenin has been proved as a potential therapy for allergic skin diseases and bronchial asthma treatments in combination with Fluticasone by reducing its dose from 50 to 25 μg/mice in combination to circumvent the long term side effects of Fluticasone. The beneficial effect of Hecogenin may be related to the diminution of TNF-α and IL-12 cytokines production in Balb/c mice.

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Hecogenin; fluticasone; ovalbumin; 2,4-dintroflurobenzene; pro-inflammatory cytokines; histopathology

Introduction

Atopic dermatitis (AD) is an inflammatory skin disease in human being and animals represents with rash and severe itching. It is caused by a multifaceted inter-relationship among environmental, genetic, immunologic, pharmacologic, psychological, and skin barrier dysfunction factors [1]. The occurrence of AD is 1–3% in adults and 10–20% in children and nowadays, the prevalence goes on increasing [2,3]. The typical allergens responsible for AD are several organic compounds that contain metal ions such as nickel and chromate that penetrate the skin and bind covalently or by forming complex with proteins such as hapten [4].

Asthma is a complex chronic lung disease characterized by airway inflammation, reversible constriction of airway smooth muscles, airway edema, lung eosinophilia, increased mucus secretion by goblet cells, an elevated serum IgE level and airway hyper-responsiveness (AHR) to variety of spasmogens [5]. According to the World Health Organization, nearly 235 million people currently suffer from asthma in today's life. This asthmatic situation is very common among pediatric age group. In India, for asthma nearly 57,000 deaths were reported in 2004 and it was considered as one of the most important cause of morbidity and mortality in rural India [6]. The constriction of airways smooth muscle and development of AHR are important pillars of bronchial asthma [7]. The natural products (plant origin) have been found to be an admirable resource of novel active substances [8,9].

Hecogenin (HG) (Figure 1) is obtained from the leaves of species such as *Agave* genus, including *A. cantala*, *A. sisalana*, *A. avellanidens*, *A. cerulata, A. cocui*, *A. goldmaniana*, and *A. aurea* [10]. It is used as a principal raw material for the synthesis of steroidal drugs. The extracts obtained from above plants have been used for their cardioactive, larvicidal [11], hypotensive, antifungal [12], and anti-ulcer activity [13]. Cerqueira et al. have studied the effect of HG on lipid peroxidation, oxidative stress, and myeloperoxidase (MPO) an important biomarker of inflammation. Their protective effects have been confirmed by histopathological analysis and COX-2 immunohistochemistry studies of rat gastric mucosa [14]. The commonly used anti-dermatitis and anti-asthmatic therapy is glucocorticosteroids (GCs). However, these drugs are frequently linked to severe adverse effects [15,16]. Many physicians and health care providers have reported that long-term GC treatment leads to adverse effects and is dose

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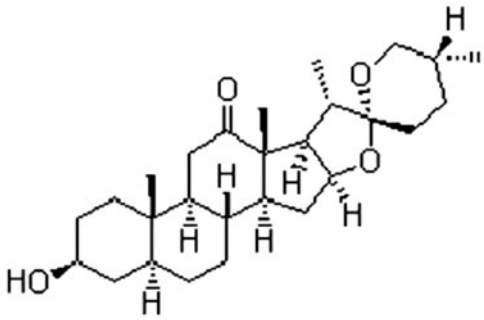

Figure 1. Chemical structure of hecogenin.

dependent. The long-term use of low dose GCs can be a feasible therapeutic alternative for treatment of patients of inflammatory disorders [17,18].

Fluticasone (FC) propionate is a topically active corticosteroid molecule used as a standard for the research study [19]. FC at high dose concentrations interacts with DNA recognition sites to activate transcription through increased histone acetylation of anti-inflammatory genes and transcription of several genes linked to glucocorticoid side effects (transactivation). Many physicians and health care providers had a problem with the long-term FC treatment because of their well-known adverse effects such as metabolic disorders, diabetes, hypertension, gastro-intestinal irritation, ulcers, glaucoma, and bone marrow suppression associated with high doses [20]. FC also has post-transcriptional effects and then decreases stability of some pro-inflammatory mRNAs [21].

Hence, the rationale behind such a research work was to minimize the dose of synthetic glucocorticoid by combining FC with HG. Our earlier research study has shown the anti-inflammatory effects of HG and its combination with FC on croton oil induced ear edema in mice, cotton pellet induced granuloma and TNBS-induced colitis in rats through inhibition of pro-inflammatory cytokines such as TNF-α and IL-12 [22]. In the present study, we have investigated the effect of HG and HG + FC on DNFB induced dermatitis and ovalbumin (OVA) induced AHR in Balb/c mice by studying its effects on ear thickness, ear weight, erythema score, total and differential cell count in BAL fluid, MPO, serum cytokines, and histopathological analysis of ear and lung tissue.

Materials and methods

Drugs and chemicals

HG was purchased from TCI, China (Causeway Bay, Hong Kong). 1-Fluoro-2,4-dinitrobenzene (DNFB), OVA, hexa-decyl-trimethyl-ammonium-bromide (HTAB) and O-dianisidine hydrochloride were purchased from Sigma Aldrich (St. Louis, MO, USA). FC was obtained as a gift sample from Sun Pharma (Vadodara, India). Cytokines kits for estimation of TNF-α, IL-12, IL-6, and TXB_2 were purchased from Krishgen Biosystem, Pvt. Ltd. (Mumbai, India). All other reagents and chemicals used in this experiment were of analytical grade and procured from local suppliers.

Table 1. Grouping of animal in DNFB-induced AD in Balb/c.

Groups	Drugs	Dose (topical)
Group I	Control	20 µl of acetone
Group II	DNFB	50 µL in (AOO, 4:1)
Group III	HG	20 µl of 50 µg/mice
Group IV	FC	20 µl of 50 µg/mice
Group V	HG + FC	20 µl of 25 µg/mice, each

Table 2. Grouping of animals in OVA-induced AHR in Balb/c.

Groups	Drugs	Dose (intra-nasal)
Group I	NC	20 µl of phosphate buffered saline
Group II	OVA	50 µg for sensitization and 2.5% w/v for challenge
Group III	HG	20 µl of 50 µg/mice
Group IV	FC	20 µl of 50 µg/mice
Group V	HG + FC	20 µl of 25 µg/mice, each

Experimental animals

Six female Swiss albino mice and male Balb/c mice (20–25 g) were procured from National Institute of Biosciences (NIBS), Pune. Mice were housed under specific pathogen free conditions with a 12 h light/dark cycle and free access to standard rodent food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Sinhgad College of Pharmacy (Pune, India) (SCOP/IAEC/2014-15/202).

Acute toxicity study

The acute oral toxicity study was carried out in female Swiss albino mice (20–25 g) as per OECD-425 guidelines. The animals were fasted for 3 h prior to the experiment. The animals were administered with a single dose of HG (2000 mg/kg) and were observed for their mortality during the 48 h study period (short term) toxicity [23].

Selection of HG and FC dose

The research work was carried out to minimize the side effects of FC by combining FC with phytosteroid HG. The dose of HG (50 µg/mice) and HG + FC (25 µg/mice, each) was chosen on the basis of pilot study. Primarily, the dose finding study was performed by selecting various doses of HG such as 50, 75, and 100 µg/mice and all doses exhibit significant anti-inflammatory activity in mice. Hence, 50 µg/mice dose of HG was preferred for further anti-inflammatory activity.

Grouping of animals in DNFB induced AD and OVA induced AHR

The Balb/c mice were divided into five groups ($n = 6$). The grouping of animals was as shown in Tables 1 and 2, respectively.

Induction of AD and experimental design

The AD was induced in accordance with the experimental procedure of Kim et al. [24]. AD-like skin lesions were induced by the repeated application of 25 µL of 0.15% DNFB

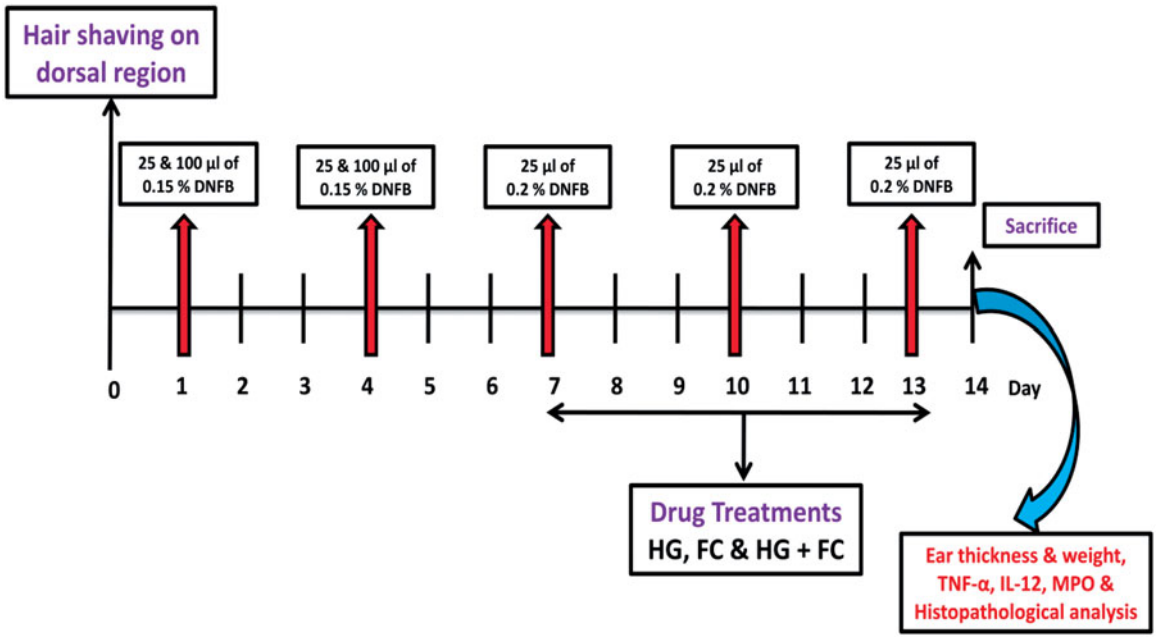

Figure 2. Schematic representation of DNFB induced AD in Balb/c mice.

in acetone/olive oil (AOO) (3:1) to inner and outer ears surfaces and 100 µL was applied to shaved back skin on days 1 and 4, once daily. On 7, 10, and 13 days, sensitized mice were challenged by applying 0.2% DNFB to back and ear skin surfaces. On day 7–13, drug treatment HG (50 µg/mice), FC (50 µg/mice), and HG + FC (25 µg/mice, each) was done. NC mice were treated with the same volumes of acetone [24]. The experimental design of DNFB induced AD is shown in Figure 2.

Measurement of ear thicknesses and ear weights in AD

The degree of ear thickness and swelling were used for the evaluation of AD. The measurement of ear thickness and weights were performed by standard method of Kim et al. [24]. On day 14, the animals were sacrificed with high dose of anesthetic ether and the ear thicknesses was measured with Vernier caliper. The weights of left and right ear pieces (5 mm) were determined.

Measurement of erythema score in AD

Depending on the rigorousness of erythema/hemorrhage, edema, excoriation/erosion, and dryness/scaling, each sign was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The assessment was done by a blind investigator who did not know the grouping of animals. The sum of the individual scores was defined as the erythema score [25].

Induction of AHR and experimental design

Male Balb/c mice (20–25 g) were intra-peritoneally injected with a mixture of OVA (50 µg) and alum (1 mg) in 0.2 mL of normal saline solution except for NC group on 1 and 7 days. The mice were challenged with 2.5% (w/v) OVA aerosol on 14 and 21 days, through a nebulizer for 20 min (0.5–5 µ particle size and pressure range 30–36 psi). The NC mice were exposed to saline aerosol for 20 min on 14 and 21 days 1 h before each OVA sensitization and challenge on 14 and 21 days after the initial sensitization [26]. Twenty microliters of HG (50 µg/mice), FC (50 µg/mice), and HG + FC (25 µg/mice, each) were administered by intra-nasal route once daily from days 14 to 21, respectively [27]. Animals were sacrificed 48 h after the last challenge on day 22 to characterize the anti-inflammatory effects of HG and HG + FC. The experimental design is as shown in Figure 3.

Collection of bronchoalveolar lavage fluid (BALF)

The mice were anesthetized with anesthetic ether after 24 h of last challenge. A tracheal cannula was inserted via mid cervical incision and lavaged twice with 1 mL of ice-cold phosphate-buffered saline (PBS) of pH 7.4 [28].

Total and differential cell count

The BAL fluid was subjected to centrifugation at 170×*g* for 10 min at 4 °C. The supernatant was removed and pellets obtained after the centrifugation were resuspended in 0.5 mL of PBS. The total and differential leukocyte count was done by using hematology autoanalyser [28].

Estimation of MPO in ear and lung tissue

The frozen, isolated ear, and lung tissue samples were weighed, washed twice in phosphate buffer (pH 6.0 at 4–8 °C), homogenized in a solution containing 0.5% HTAB dissolved in 50 mM potassium phosphate buffer (pH 6). The samples were then centrifuged at 10,000 rpm for 20 min at 4 °C. The samples were freeze-thawed three times and sonicated for 20 s. An aliquot of 0.1 mL supernatant or standard was then allowed to react with 2.9 mL solution of 50 mM

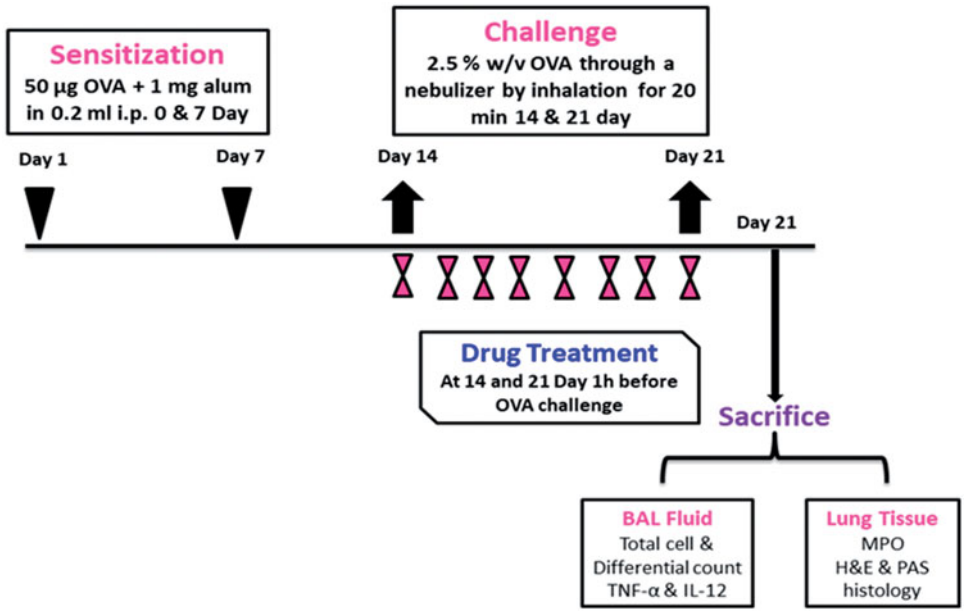

Figure 3. Schematic representation of OVA induced AHR in Balb/c mice.

potassium phosphate buffer at pH 6 containing 0.167 mg/mL of O-dianisidine hydrochloride and 0.0005% hydrogen peroxide (H_2O_2). After 5 min, the reaction was stopped with 0.1 mL of 1.2 M hydrochloric acid. The rate of change in absorbance was measured using a spectrophotometer at 460 nm. MPO activity was expressed in milli unit (mU) per gram weight of wet tissue [29].

Measurement of serum TNF-α, IL-12, IL-6, and TXB_2 levels

Mice were anesthetized with anesthetic ether. The blood was collected from retro-orbital plexus and centrifuged at 3000 rpm for 10 min at 4 °C, the supernatants were stored at –80 °C for analysis. The estimations TNF-α, IL-12, IL-6, and TXB_2 were performed by using ELISA kits according to the manufacturer instructions (Krishgen Biosystem, Mumbai, India).

Histopathology of ear and lung tissue

The ear and lung tissue was stored in buffered formalin 10% (v/v). The paraffin embedded blocks were cut into 5 µm sections using a microtome mounted and stained with hematoxylin and eosin (H&E) for routine histology, toluidine blue for immune cell infiltration and mast cells and Giemsa stain for goblet cell study [30].

Statistical analysis

The data were expressed as mean ± SEM. Statistical analysis was carried out by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. p Value $<.05$ was considered statistically significant.

Results

Acute toxicity study

The acute oral toxicity study was carried out in accordance with OECD guideline 425. In this study, none of the mice have shown observable signs of toxicity and mortalities upon single dose of HG (2000 mg/kg) on day one.

Effect of HG and its combination on ear thickness and ear weight

The representative photographs of mice from five different groups and ear tissue samples are shown in Figure 4. The DNFB induced increase in ear thickness and weight was significantly inhibited by topical application of HG (50 µg/mice) (thickness and weight $p<.001$) and HG + FC (25 µg/mice, each) (thickness and weight $p<.001$). Whereas, the treatment of mice with FC (50 µg/mice) (thickness and weight $p<.001$) also significantly decreased the DNFB induced ear thickness and weights as shown in Figure 5.

Effect of HG and its combination on erythema score

The ear erythema score of DNFB treated mice was found to be 4 ($p<.001$) which was compared to erythema score of NC mice having 0 score. The treatment of mice with HG (Score = 2; $p<.001$), FC (Score = 2; $p<.001$), and HG + FC (Score = 1; $p<.001$), was significantly inhibited the erythema score as compared to DNFB treated mice (Figure 6).

Effect of HG and its combination on total and differential cell count

The Balb/c mice were immunized with OVA and subjected to two OVA aerosol challenges showed statistically significant ($p<.001$) increase in total cells (TCs), monocytes (MCs),

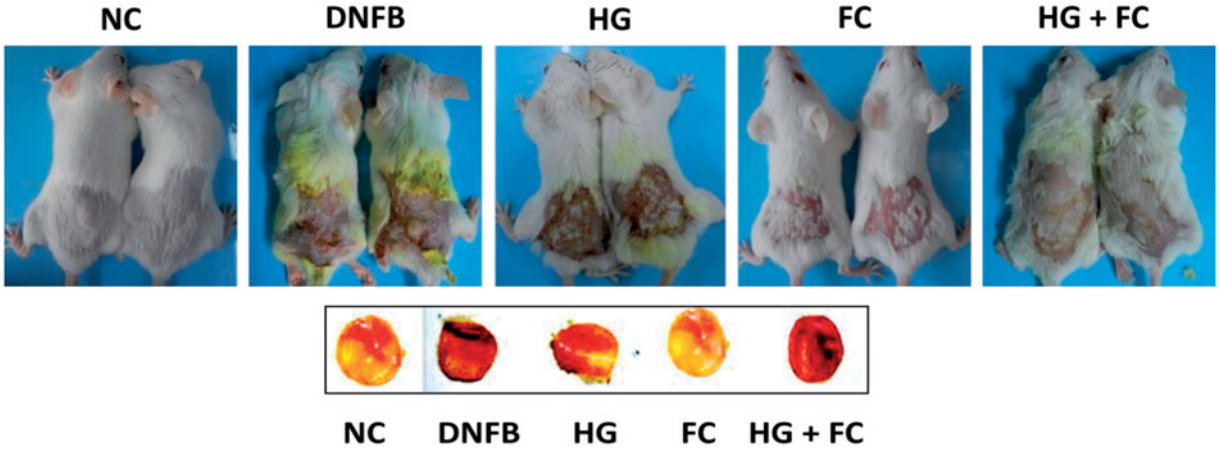

Figure 4. Effect of HG and HG + FC in DNFB induced AD in Balb/c mice. Representative photographs of each group of mice and ear tissue samples obtained after completion of DNFB model. Six animals were allocated to each group.

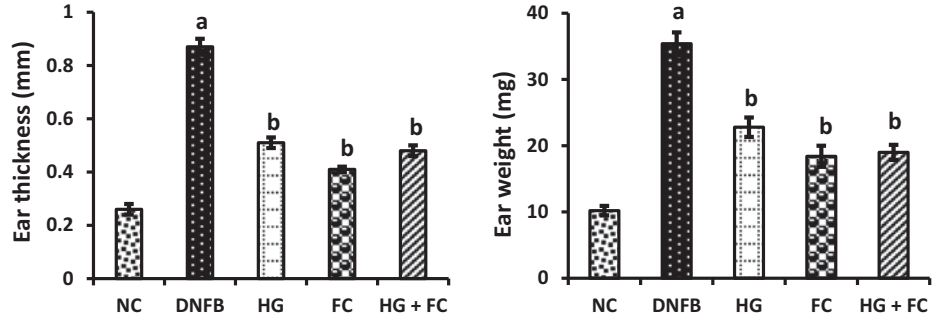

Figure 5. Effect of HG and HG + FC on ear thickness (mm) and ear weight (mg) in DNFB induced dermatitis in mice. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's test. [a]$p<.001$ represents the significance level when compared with NC group and [b]$p<.001$ when compared with DNFB.

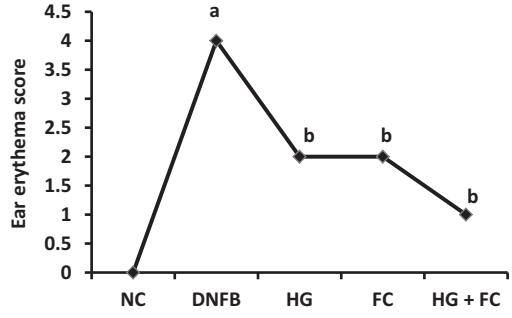

Figure 6. Effect of HG and HG + FC on ear erythema score (0–4) in DNFB induced dermatitis in mice. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's test. [a]$p<.001$ represents the significance level when compared with NC group and [b]$p<.001$ when compared with DNFB.

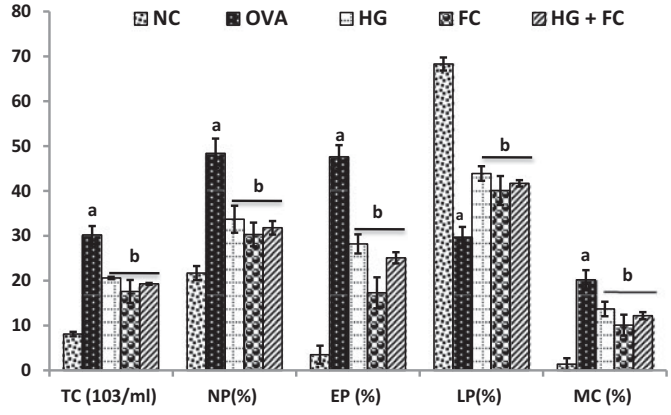

Figure 7. Effect of HG and HG + FC on total and differential cell count in OVA induced AHR in Balb/c mice. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. [a]$p<.001$ represents the significance level as compared to NC group and [b]$p<.001$ as compared to OVA group.

neutrophils (NPs), eosinophils (EPs) in the BALF, whereas decrease in lymphocytes (LPs) when compared to NC mice. When compared to OVA, NC, HG, and HG + FC showed statistically significant ($p<.001$) reduction in TC count. However, the numbers of circulating NP ($p<.001$), EP ($p<.001$), and MC ($p<.001$) were significantly decreased and LP ($p<.001$) was increased by HG (50 μg/mice) and HG + FC (25 μg/mice, each) in Balb/c mice (Figure 7). While, the standard drug FC (50 μg/mice) significantly reduced the total and differential cell count in BALF in OVA induced AHR in Balb/c mice.

Effect of HG and its combination on MPO level

The representative photographs of lung tissue from each group are shown in Figure 8. The effect of HG (50 μg/mice) and HG + FC (25 μg/mice) on ear and lung MPO activity is shown in Figure 9. The MPO level in ear and lung tissue (U/g) was significantly ($p<.001$) increased in DNFB and OVA treated group when compared with NC group. When compared with DNFB and OVA group, the elevated MPO level

was significantly ($p<.001$) decreased by HG (50 μg/mice), FC (50 μg/mice), and HG + FC (25 μg/mice, each).

Effect of HG and its combination on serum TNF-α, IL-12, IL-6, and TXB_2 level

The treatment of animals with DNFB and OVA had significantly ($p<.001$) elevated levels of proinflammatory cytokines TNF-α, IL-12, IL-6, and TXB_2 as compared to NC mice. Whereas, the animals treated with HG (50 μg/mice), FC (50 μg/mice), and HG + FC (25 μg/mice, each) showed significant ($p<.001$) decrement in the TNF-α, IL-12, IL-6, and TXB_2 level as compared to DNFB and OVA treated animals respectively as shown in Figures 10 and 11.

Effect of HG and its combination on histopathology of ear and lung tissues

In a histological analysis of ear tissue on H&E staining, no abnormal changes in the NC mice (Figure 12(A)) were shown, whereas DNFB challenge to mice induced severe edema and accumulation of inflammatory cell infiltration in the epidermis and dermis of ear tissue (Figure 12(B)). The treatment of mice with HG and HG + FC showed restoration of increased inflammatory cell infiltration and ear thickness induced by DNFB challenge (Figure 12(C,E)). The FC group mice could display almost normal histopathological features and showed accumulation of only few immune cells (Figure 12(D)).

In the present study, we have studied the effect of HG and its combination on the infiltration and degranulation of mast cells, using toluidine blue staining. The numbers of mast cells in the ear dermis tissue were significantly increased by DNFB challenge (Figure 12(B)) as compared to NC mice (Figure 12(A)). The degranulated mast cells were significantly reduced in the dermis region of HG treated mice and HG + FC (Figure 12(C,E)) as compared to DNFB treated mice. The FC group displayed almost normal features and few mast cells infiltration were found in the ear dermis tissue (Figure 12(D)).

The histology of bronchi after staining with H&E and Giemsa stains revealed the normal structural design of mucus glands with mild degree of peribronchial inflammation and active goblet cells in NC mice (Figure 13(A)). Whereas, in OVA, challenged mice showed severe increase in peribronchial inflammation, epithelial fragility, wall thickening and metaplastic goblet cells (Figure 13(B)). The treatment of mice with HG and HG + FC shows restoration in the bronchial changes which occur in the bronchi when compared with OVA control (Figure 13(C,E)). However, similar observation was seen in mice treated with FC (Figure 13(D)).

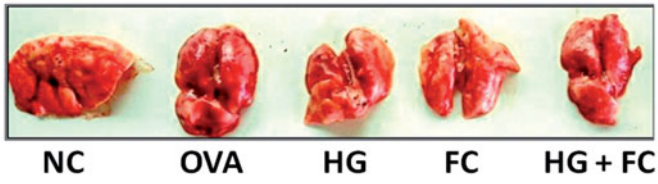

Figure 8. Representative photograph of lung tissue in each group in OVA induced AHR in Balb/c mice taken after completion of study.

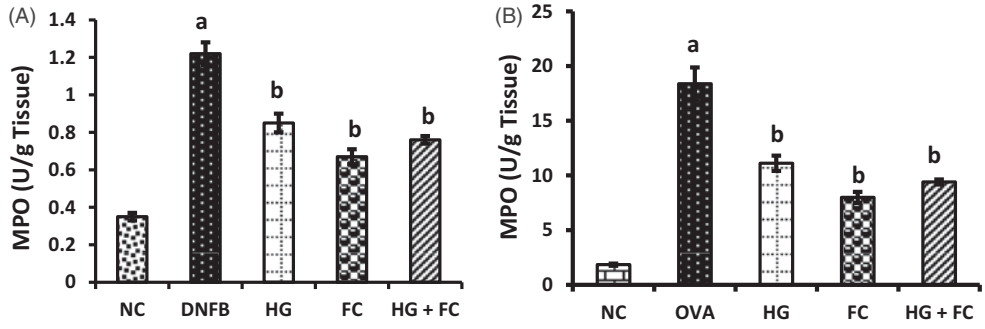

Figure 9. Effect of HG and HG + FC on MPO level in A = DNFB induced dermatitis and B = OVA induced AHR in Balb mice. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's test. [a]$p<.001$ represents the significance level when compared with NC group and [b]$p<.001$ when compared with DNFB and OVA.

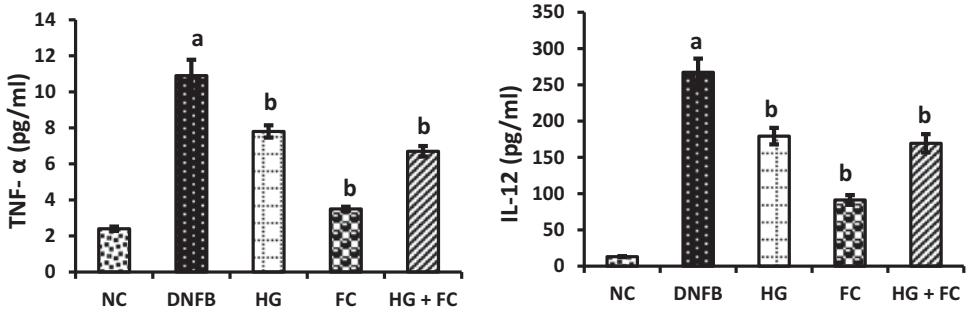

Figure 10. Effect of HG and HG + FC on TNF-α and IL-12 (pg/mL) in DNFB induced dermatitis in Balb/c mice. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's test. [a]$p<.001$ represents the significance level when compared with NC group and [b]$p<.001$ when compared with DNFB.

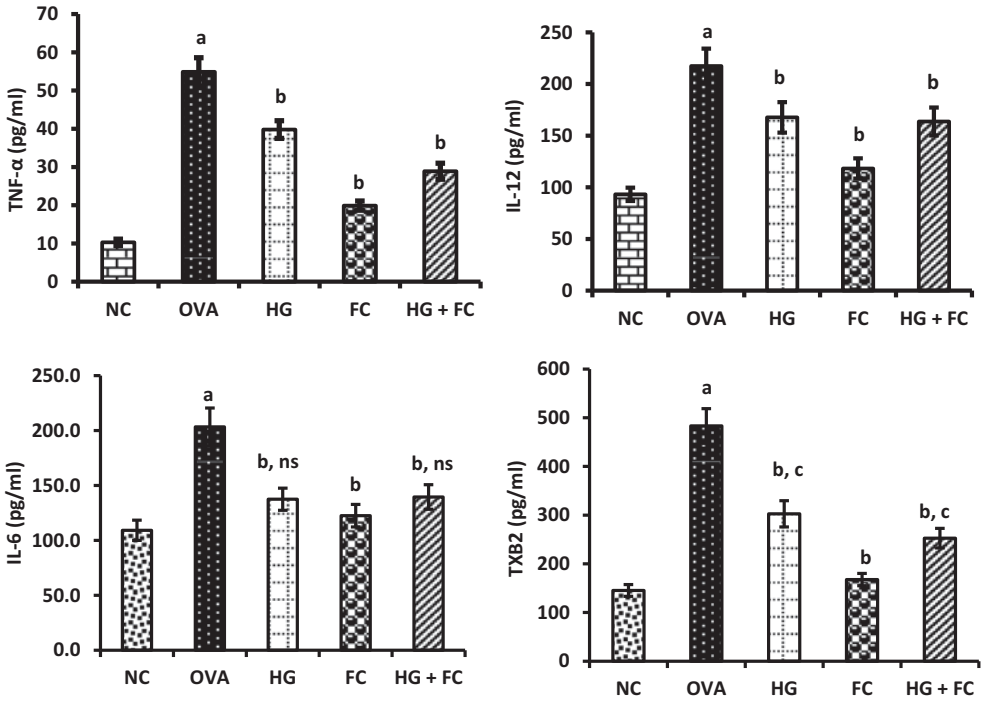

Figure 11. Effect of HG and HG + FC on TNF-α, IL-12, IL-6, and TXB_2 (pg/mL) in OVA induced AHR in Balb/c mice. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's test. [a]$p < .001$ represents the significance level when compared with NC group and [b]$p < .001$ when compared with OVA.

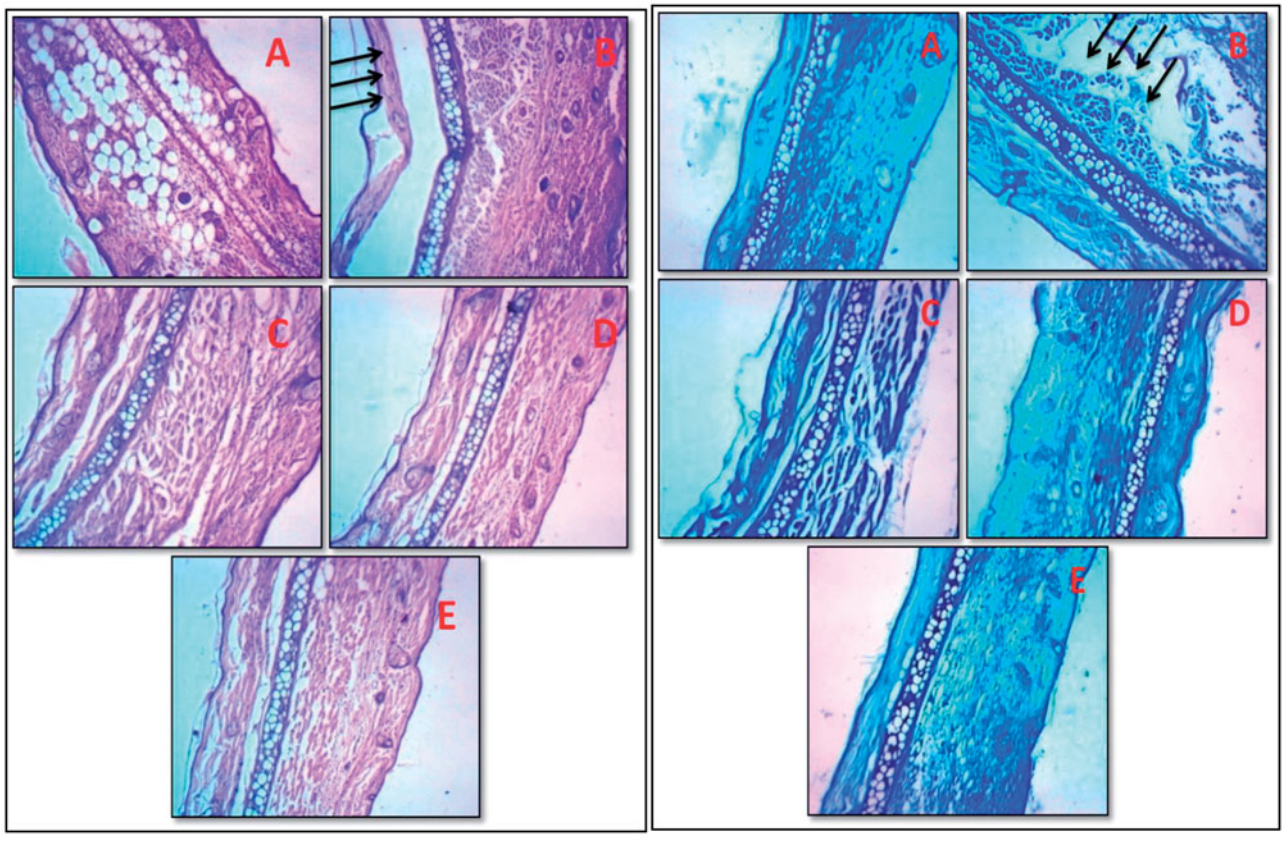

Figure 12. Photomicrograph of transverse sections of ear tissue sensitized with DNFB stained with H&E and toluidine blue examined under light microscopy (100×). Treatments: normal control (acetone) (A), DNFB 0.2% DNFB (B), HG (50 μg/mice) (C), FC (50 μg/mice) (D), and HG + FC (25 μg/mice, each) (E). The shown sections are representative of six animals per group.

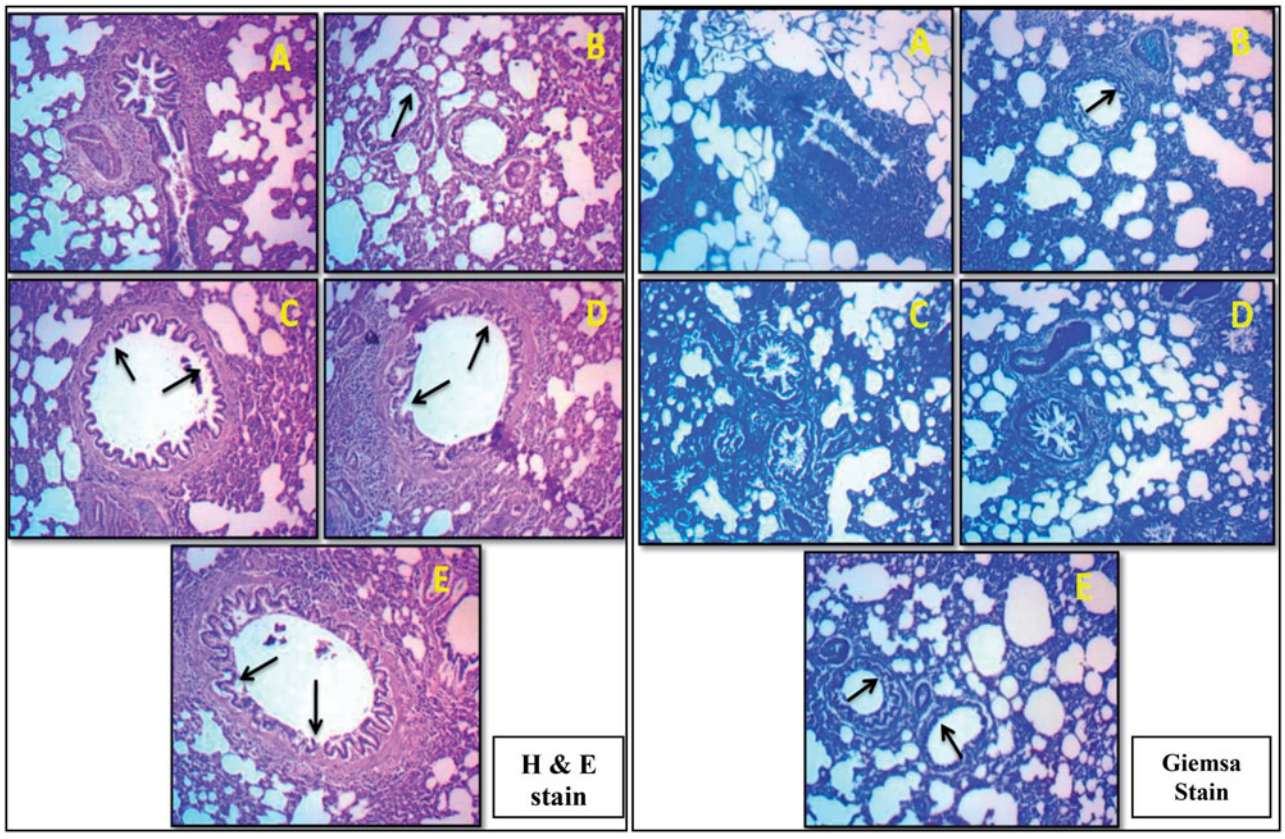

Figure 13. Photomicrograph of transverse sections of lung tissue sensitized with OVA stained with H&E and Giemsa staining examined under light microscopy (100×). Treatments: normal control (normal saline) (A), OVA 2.5% w/v OVA (B), HG (50 μg/mice) (C), FC (50 μg/mice) (D), and HG + FC (25 μg/mice, each) (E). The shown sections are representative of six animals per group.

Discussion

AD is a common skin disease, characterized by edema, erythema, excoriation, and scaling of skin [31,32]. Topical steroidal therapy is very successful for the treatment of AD and bronchial airway inflammation but, because of its potential adverse effects it cannot be used for long-term use (e.g. skin atrophy and hyperpigmentation of skin) and shows resistance to corticosteroids therapy in bronchial asthma [33]. Therefore, it is necessary to minimize the corticosteroid dosage schedule. Owing to the safety and low cost, herbal medicines can be an effective drug therapy for treatment of AD and AHR in patients [34]. Therefore, we have demonstrated the anti-inflammatory effects of HG and HG + FC on AD like skin lesions and OVA induced AHR in Balb/c mice.

In our animal model, repeated application of DNFB causes increase in ear thickness, ear weight, hyperplasia, edema, and spongiosis in mice (Figures 4 and 5). Moreover, the levels of TNF-α and IL-12 were elevated by DNFB in inflamed tissues (Figures 11 and 12). The treatment of mice with HG and HG + FC effectively prevented the ear thickness and ear weight, as well as hyperplasia, edema, and spongiosis in inflamed tissues. Contact of skin with the hapten induces the release of TNF-α, which is another Th1 cytokine, and IL-l2 during the sensitization phase [35]. Furthermore, TNF-α exerts a stimulatory effect on skin resident cells, resulting in enrollment of leukocytes during hypersensitivity responses [36]. Presently, HG and its combination with FC effectively reduced the production levels of TNF-α and IL-12 in inflammatory tissues.

The measurement of MPO activity reveals the degree of cell damage and level of cell injury in the mice ear tissue [37]. MPO level was significantly increased in the mice ear tissue treated with DNFB challenge when compared to NC mice. Topical treatments of mice with HG and its combination have significantly inhibited the MPO activity, indicating that these compounds may influence migration of various inflammatory cells in the inflammatory process [38]. The contact of skin with hapten causes release of TNF-α and IL-12 during the DNFB sensitization phase [35]. Therefore, TNF-α and IL-12 are the important mediators of cutaneous inflammatory response [39,40]. Furthermore, TNF-α also exerts a stimulatory effect on skin resident cells, resulting in leukocytes enrollment during contact hypersensitivity responses [36]. In the present study, the levels of TNF-α and IL-12 were elevated by DNFB challenge in inflamed ear tissues. Moreover, HG and HG + FC treatment in mice effectively reduced the production of TNF-α and IL-12 in inflammatory tissues. These data indicate that HG is an anti-inflammatory agent by its Th1 skewing reaction, resulting in diminution of inflammatory reactions (hyperplasia and spongiosis) and immune cell infiltration. The histopathological studies revealed that DNFB challenge increased the dermal thickening, hyperplasia, presence of inflammatory cells, increase in number and size of mast cells in DNFB group. All these

above changes were effectively restored by treatment of mice with HG and HG + FC effectively.

Asthma is an airways inflammatory disease that can be exacerbated by numerous extrinsic factors, such as allergens [41]. OVA sensitization and subsequent OVA challenge in a murine model induce the infiltration of immune cells, including EPs and LPs in the BALF and mucus secreting goblet cell hyperplasia [42]. In OVA induced AHR model, the secretion of pro-inflammatory cytokines by activated mast cells, T-LPs and injured bronchial cells may be responsible for the pathogenesis of AHR in bronchial asthma [43]. In the present study, HG and its combination were investigated for their *in vivo* anti-asthmatic activity on OVA induced AHR in Balb/c mice.

Since the migration of inflammatory cells into the lungs is inevitable in allergic and asthmatic disorders; it has been found that there was a significant increase in the number of TC and differential count in BALF of OVA challenged mice. The results revealed that HG and HG + FC show significant reduction in TC count as compared to OVA challenged mice. In differential cell count, the increased number of EPs and NPs has been observed. HG has the potential of attenuating the proliferation and transmigration of EPs and NPs in the lung that plays important role in allergic asthma [44,45]. The increased number of EPs shows the phenomenon of eosinophilic infiltration and the increased number of NPs leads to activation of interleukin-8 which induces sputum secretion during allergic asthmatic condition. The result revealed that HG and HG + FC proved to be more potent in reducing the EP and NP count as compared to HG alone. From the results, it could be suggested that HG and HG + FC dampens the asthmatic inflammation in lung by its action on EPs.

The important parameter estimated with the lung homogenate was MPO. The elevated level of MPO has been observed in lung tissue of OVA sensitized mice which was significantly decreased by HG and HG + FC that indicate the cellular damage leading to altered cellular function within the inflamed lung. The elevated level of TNF-α, IL-12, IL-6, and TXB_2 in the lung of Balb/c mice reflects the activation of pro-inflammatory response whereas, treatment of mice with HG and HG + FC significantly inhibits these levels may be virtue of its anti-inflammatory potential.

This has been further strengthened by histopathological analysis of ear and lung tissue results obtained from OVA sensitized model. These results showed structural changes in the asthmatic bronchi airways which included goblet cell hyperplasia, metaplasia, epithelial fragility, enlarged mucus glands, angiogenesis and increased matrix deposition in airway wall, increased airway smooth muscle mass, thickening and elastin abnormalities of the bronchial wall. The histology of lung tissue stained with H&E and Giemsa reveals that HG and HG + FC were effective and shows restoration of bronchial airways in Balb/c mice.

Conclusions

This study demonstrated that the topical application of HG was able to inhibit AD symptoms in DNFB model and AHR in OVA treated Balb/c mice. The anti-inflammatory activity of topically applied HG may be due to down regulation of cytokines such as TNF-α, IL-12, IL-6, TXB_2, and MPO levels. These decreased levels of pro-inflammatory cytokines resulting in reduced hyperplasia of ear tissue, edema, epidermal spongiosis, and immune cell infiltration. These consecutive anti-inflammatory reactions of HG and its combination with FC finally led to the inhibition of ear thickness and swelling. In OVA induced AHR model, HG and its combination treatment reduces the accumulation of inflammatory cells in BALF of mice. Furthermore, HG + FC was found to be more potent than HG in all aspects. Our research outcomes suggest that HG can serve as a potential therapeutic target for the treatment of AD and AHR in Balb/c mice so, it can be used to reduce or replace the corticosteroids usage.

Disclosure statement

No potential conflict of interest was reported by the authors.

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