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Asthma-Alleviating Potential of 6-Gingerol: Effect on Cytokines, Related mRNA and c-Myc, and NFAT1 Expression in Ovalbumin-Sensitized Asthma in Rats

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ABSTRACT: In this study, we aimed at assessing the therapeutic potential of 6-gingerol ([5S]-5-hydroxy-1-[4-hydroxy-3-methoxyphenyl]-3-decanone) against ovalbumin-sensitized asthma in rats. The rats were treated intraperitoneally with 6-gingerol (75 mg/kg body weight) for 30 days and a theophylline (200 mg/kg body weight)-treated group as a control. Changes in the levels of T-cell-linked cytokines (interleukin-4 [IL-4], IL-5, IL-13, and interferon-gamma [IFN- γ]), total immunoglobulin E (IgE), gene expressions of bitter taste-sensing type 2-receptor 10 (T2R10), inositol 1,4,5-triphosphate receptor 1 (IP3R1), Orai1 and protein expressions of nuclear factor of activated T cells 1 (NFAT1), c-Myc and histopathological changes were observed in rats. 6-Gingerol exerts its beneficial impacts like theophylline in lessening IL-4, IL-5, and IL-13, and IgE and increasing the level of IFN- γ . Significant down-regulation of T2R10 gene expression and up-regulation of IP3R1 and Orai1 gene expression were observed in experimental rats and these alterations were normalized after treatment with 6-gingerol or theophylline. The histopathological study revealed that the accumulation of glycoprotein and thickness of alveolar epithelium in asthmatic rats and supplementation with 6-gingerol or theophylline in asthmatic rats restored these changes to normal. In conclusion, 6-gingerol has a protective effect on lungs in ovalbumin-sensitized asthma in rats.

KEY WORDS: 6-gingerol, asthma, natural products, lung disorders

I. INTRODUCTION

Asthma is a persistent provocative disease categorized by bronchial hyper-responsiveness, airway inflammation and remodeling, mucus hypersecretion, and variable airflow obstruction.¹ The prevalence of asthma is rapidly rising around the globe, particularly among children, and it has become a major cause of morbidity and mortality in developed countries.² Cumulative data show that the worldwide occurrence of allergic asthma has increased considerably over the decades, especially in developed countries.³ Bronchial asthma may be aggravated by

factors such as dust, cold air, allergens, chemicals, and respiratory infection principal to stimulation of inflammatory cells and penetration of cell-derived mediators such as interleukins, eosinophils, neutrophils, immunoglobulin-E (IgE), lymphocytes, and histamine into the bronchial lumen.⁴ Interestingly, recent studies have sustained the concept that a promising strategy to cure asthma is the anti-inflammatory approach, but there are some data suggesting that the treatment of asthma should be through the controlled progression of the airway inflammatory reaction.⁵

Nebulized and inhaled racemic adrenaline typically used across the world appears to be (clinically) effective in patients with asthma,⁶ but β_2 -agonists have apparent disputed symptomatic effects.⁷ The regular use of β_2 -agonists results in the issues of bronchoprotective and bronchodilator tolerance. After β_2 -agonist therapy, all patients achieved an absolute reduction of catarrh, cough, and asthma, but masked inflammation and allergen responsiveness were not enhanced.⁸ Even though conservative anti-asthmatic drugs have been broadly used for the management of bronchial asthma, they only treat the symptoms and these drugs possess more local and systemic side effects.⁹ Conversely, in the past few decades, a number of drugs from native plant sources have been investigated for their anti-asthmatic properties and have fewer or no side effects.^{10,11} These qualities necessitate the production of new anti-asthma agents using these sources. Ginger, the rhizome of *Zingiber officinale* Roscoe, has been known as a flavor and conventional medicine for many years. However, 6-gingerol ([5S]-5-hydroxy-1-[4-hydroxy-3-methoxyphenyl]-3-decanone), one of the major pungent constituents of ginger, is revealed to have various interesting pharmacological effects,¹² but no studies have yet specifically addressed the efficacy of 6-gingerol against asthma. The present study addresses the protective effect of 6-gingerol against ovalbumin-sensitized asthma in experimental rats.

II. MATERIALS AND METHODS

A. Test Materials

Commercially available purified 6-gingerol [$C_{17}H_{26}O_4$] (catalog #23513-14-6) (Fig. 1) from ginger (99%) was obtained from Natural Remedies (Bangalore, India). Theophylline (catalog #T1633)

was purchased from Sigma-Aldrich (Bangalore, India). All other reagents used in the experiments were of analytical grade and of the highest purity.

B. Animals

Male Wistar rats (6-8 weeks of age) were obtained from the Department of Biochemistry, Muthyammal College of Arts and Science (Rasipuram, Namakkal district, Tamil Nadu, India). Experimental animals were kept under standard laboratory conditions (temperature $22 \pm 2^\circ\text{C}$; humidity 40–60%) and permitted food and water *ad libitum*. Rats, weighing 180–200 g, were first separated into four groups of six each ($n = 6$). All procedures involving laboratory animals were performed in accordance with the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India, and the study was approved by the institutional animal ethical committee (IAEC) of the Muthyammal College of Arts and Science (Approval No: IAEC/MCAS/09/2016).

C. Induction of Asthma in Rats

Briefly, 1 mg of ovalbumin (OVA) and 200 mg of aluminum hydroxide (Sigma-Aldrich) were injected intraperitoneally on days 1 and 8. From day 14, the animals were placed in a closed chamber (dimensions: $30 \times 20 \times 20$ cm) exposed to aerosol particles of 4% OVA for 17–19 days for 15 min daily using a nebulizer (CX3; Omron Health Care Europe B.V., the Netherlands). The rats of the normal groups were treated similarly with saline instead of the OVA solution. Rats are easily sensitized using OVA and show immediate and late sensitization responses after an allergen challenge.¹³

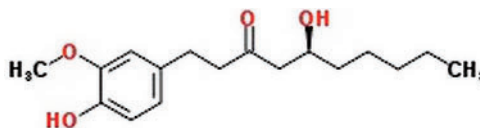


FIG. 1: Structure of 6-gingerol

D. Experimental Design

After the successful induction of experimental asthma, the rats were divided into four groups comprising a minimum of six rats each as follows: Group 1: normal control rats; Group 2: asthma control rats; Group 3: OVA-induced asthma rats treated with 6-gingerol (75 mg/kg body weight)¹² in vehicle solution intraperitoneally with insulin needle once a day for 30 days; and Group 4: OVA-induced asthma rats treated with theophylline (200 mg/kg body weight)¹⁴ in vehicle solution intraperitoneally with an insulin needle once a day for 30 days.

The test drug and reference standard drugs were supplemented intraperitoneally for 30 days after sensitization/challenge phase with OVA. Groups 1 and 2 rats received 0.9% normal saline solution intraperitoneally for 30 days. The experiment was terminated at the end of 30 days and then the animals were made to fast overnight, after which blood was collected by the retro-orbital sinus puncture method for the estimation. At the end of the experimental period, the rats were made to fast overnight and then anesthetized using low doses of phenobarbitone (30 mg/kg body weight) and sacrificed by way of cervical decapitation.

E. Cytokine Analysis

Diets were removed from the cages 12 h before the rats were sacrificed and 24 h after the last treatment. Blood samples were collected, centrifuged at 3000 × g for 15 min to obtain serum, and stored at 4°C until use. The levels of serum interleukin-4 (IL-4;

ab100710), IL-5 (ab204523), IL-13 (ab178014), interferon-gamma (IFN-γ; ab46025), and total IgE (ab108650) were determined by enzyme-linked immunosorbent assay. All commercially available kits used in the study were purchased from Allied Scientific Products (Kolkata, India).

F. Histopathological Study

At the end of the experiment, animals were sacrificed, lungs were harvested, and lung tissue was fixed in 10% formalin, routinely processed, and embedded in paraffin wax. Paraffin sections (5 μm) were cut on glass slides and stained with hematoxylin and eosin (H&E). They were then examined under a light microscope by a pathologist blinded to the studied groups.

G. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

At the end of the experiment, lungs were collected, samples of lung tissues were homogenized, and total RNA was extracted in the experimental groups using TRIzol reagent. cDNA synthesis was performed according to the manufacturer's protocol. The specifically designed primer pairs of T2R10, IP3R1, Orai1, and 18S used for analysis are shown in Table 1 (Shanghai Biotechnology Co., Ltd., China). In this study, the eukaryotic 18S rRNA was selected as the housekeeping gene primarily because it shows limited change in expression under different experimental conditions. The amplification reaction was conducted in a 25 μL capillary containing 2 μL of cDNA, 0.5 μL each

TABLE 1: List of primer sequences

Gene	Primer sequences (5' to 3')	Size (bp)
T2R10	AAGATGGAGGTCAATGCCAAAGG TGCATACAGCAGCAGGAAGGAGA	96
IP3R1	GAGCATTGTCACCACCTTCTTCAG GGCTTCTGGCTCGGCATCAAC	142
Orai 1	CCGTCATCGGGACGCTGCTT GATGCCACCGTTGTTGCTGCTGTT	155
18S	GAATTCCCAGTAAGTGCGGGTCATA CGAGGGCCTCACTAAACCATC	105

of the forward and reverse primers (final concentration of each, 0.5 μ M), 12.5 μ L 2 $\times$ SYBR green, and enough nuclease-free water for a final volume of 25 μ L for each reaction. The following thermal profile for RT-PCR assays was used for all primer sets: 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C, and elongation at 64°C for 25 s with the acquisition of fluorescent data.

H. Western Blot Analysis

At the end of the experiment, proteins were extracted from lungs by means of commercial kits (Thermo Fisher Scientific, New Delhi, India). Protein levels in the supernatants were determined using the BCA assay kit (Sigma-Aldrich). Each sample (60 μ g) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electrophoretic transfer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then probed with an antibody reactive through nuclear factor of activated T cells 1 (NFAT1) and c-Myc (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. After three washes with Tris-buffered saline, 0.1% Tween 20, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Then, the blots were developed by SuperSignal West Dura Extended Duration Substrate (Pierce, Waltham, MA, USA). The protein β -actin (Santa Cruz Biotechnology) was used as the loading control.

I. Statistical Analysis

All statistical analyses were performed using SPSS version 13.0 software. All data are expressed as mean $\pm$ standard deviation. One-way analysis of variance was used to detect significant differences between the control and treatment groups. Values of $p < 0.05$ were considered to be statistically significant.

III. RESULTS

A. Cytokine Analysis

Figure 2 depicts the levels of IL-4 (Fig. 2A), IL-5 (Fig. 2B), IL-13 (Fig. 2C), and IFN- γ (Fig. 2D)

in the both the control and experimental animals. Inflammatory cytokines in the asthma control group were found to be significantly increased compared with those in the normal control group ($p < 0.05$). 6-Gingerol (75 mg/kg body weight) or theophylline (200 mg/kg body weight) supplementation concealed the production of IL-4, IL-5, and IL-13 compared with those in the asthma control group ($p < 0.05$). Conversely, IFN- γ is distinguished as an indicator of Th1 cells and its level in serum was significantly reduced after induction of asthma with OVA; 6-gingerol or theophylline treatment was able to reverse this trend. In view of the antigen-specific Th2 responses triggering IgE antibody production, total IgE production in the allergic asthma rat model was examined. Total IgE levels in serum were distinctly augmented in OVA-induced asthma rats ($p < 0.05$) compared with normal control rats (Fig. 3). 6-Gingerol or theophylline administration reduced total IgE levels in serum ($p < 0.05$) compared with those in the asthma control group.

B. Histopathological Study

Histopathological study in the asthma group with H&E staining showed that the glycoprotein compounds gathered in airways, all goblet cells in bronchioles were emptied, and the height of the bronchiolar epithelium was enlarged. In addition, the thickness of epithelium in the alveoli was enhanced and many large nodules around bronchioles were observed (Fig. 4B). In the treated groups, the bronchioles and air sacs were similar to the normal control group (Fig. 4A) and the presence of lymphoid cells was less and scattered. However, the symptoms of these alterations were considerably improved in 6-gingerol- or theophylline-treated rats (Fig. 4C,D).

C. RT-PCR Analysis

The expression of T2R10 mRNA was normal in lung tissue of the normal control group, whereas it was notably diminished ($p < 0.05$) in the asthma control group. In contrast, the expressions of Orai1 and IP3R1 mRNA were considerably enhanced (p

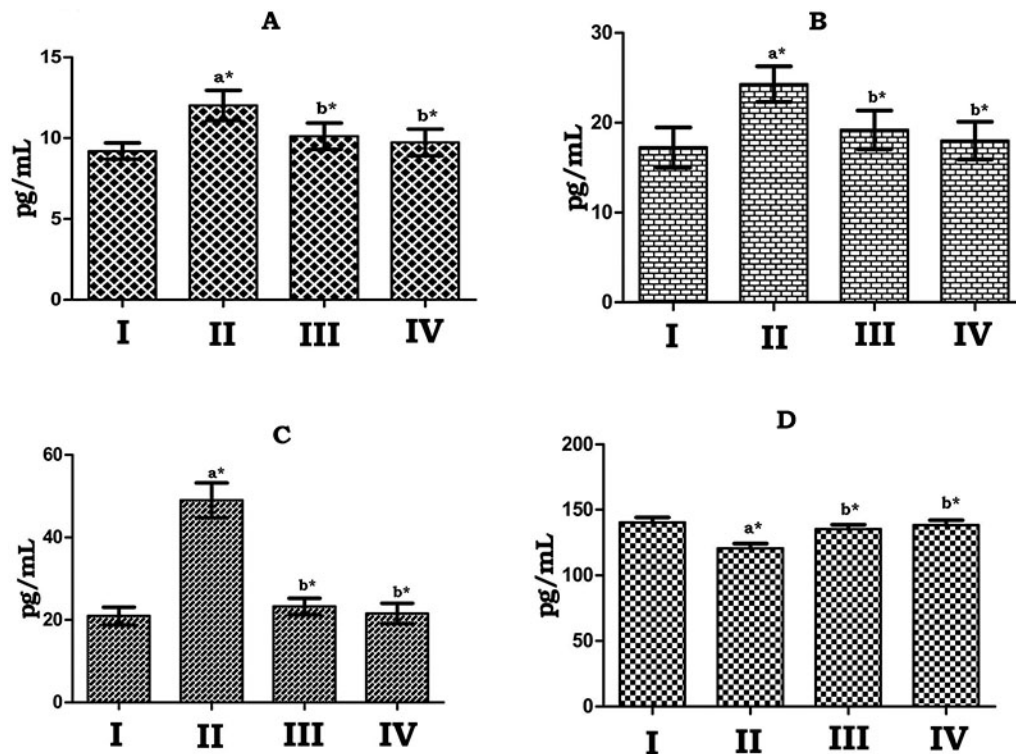


FIG. 2: (A) Effect of 6-gingerol on the level of IL-4 in control and experimental animals. (B) Effect of 6-gingerol on the level of IL-5 in control and experimental animals. (C) Effect of 6-gingerol on the level of IL-13 in control and experimental animals. (D) Effect of 6-gingerol on the level of IFN- γ in control and experimental animals. I: Normal control; II: Asthma control; III: Asthma + 6-gingerol; IV: Asthma + theophylline. Values are expressed as mean $\pm$ standard deviation for six animals in each group. Values are statistically significant at $*p < 0.05$. ^aSignificantly different from control. ^bSignificantly different from asthma control.

< 0.05) in the asthma control group compared with those in the normal control group. Treatment with 6-gingerol or theophylline reversed these changes (Fig. 5A,B), increasing the expression of T2R10 mRNA but decreasing IP3R1 and Orai1 in the lung tissues of asthma rats. There were noteworthy differences among the asthma control group and the 6-gingerol- and theophylline-treated groups.

D. Western Blot Analysis

As shown in Fig. 6A,B, the expressions of NFAT1 and c-Myc proteins were greater than before; that is, approximately 1.5-fold more than those in the normal control group. After treatment with 6-gingerol or theophylline, protein expression in the lung tissues of asthma rats was notably reduced. These

results suggest that 6-gingerol had a discernible inhibitory effect on inflammatory gene expression.

IV. DISCUSSION

OVA-induced asthma is categorized by inflammation and airway hyper-responsiveness. An animal model is suitable to study this as it imitates clinical asthma closely. These models have been used to shed light on asthma and to recognize and appraise new remedial targets.¹⁴ OVA sensitization showed airway hyper-responsiveness in rats and confirmed previous reports of bronchial hyper-reactivity development in rats.¹⁵ Glucocorticoids are the foremost drugs for controlling airway inflammation by suppressing mucus hyper-secretion. Even though mechanisms underlying the action of glucocorticoids are

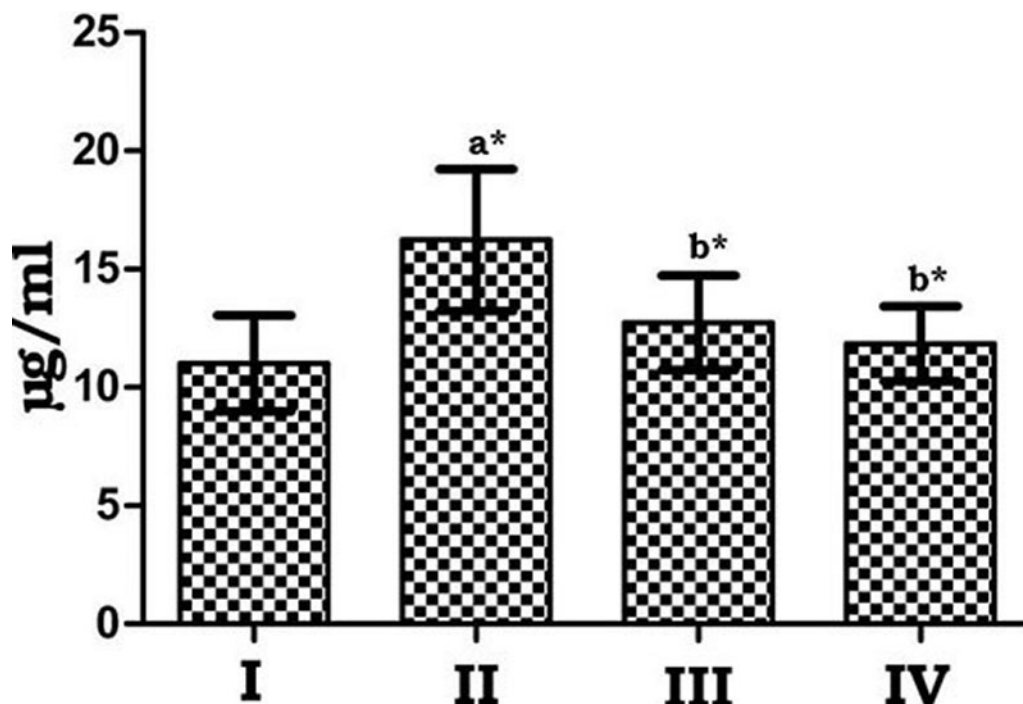


FIG. 3: Effect of 6-gingerol on the level of total IgE in control and experimental animals. I: Normal control; II: Asthma control; III: Asthma + 6-gingerol; IV: Asthma + theophylline. Values are expressed as mean $\pm$ standard deviation for six animals in each group. Values are statistically significant at $*p < 0.05$. ^aSignificantly different from control. ^bSignificantly different from asthma control.

diverse and intricate, their use is limited due to their severe adverse effects, including muscle atrophy and immune suppression.⁸ For that reason, novel therapeutic strategies are needed. In this study, using a rat model, we focused on the potential of 6-gingerol as a therapeutic agent for treating asthma.

Asthma is a persistent disease characterized by airway inflammation, erratic airflow obstruction, and bronchial hyper-responsiveness.¹⁶ It is well-known that Th1/Th2 cells disparity is the immunological basis of asthma, which is led by the principal up-regulation of Th2 cells in immune response.¹⁷ In allergic diseases such as bronchial asthma, the activity of Th1 cells (which produce IFN- γ) is condensed, whereas Th2 cell activity is activated, resulting in Th2-dominated immune responses. Abnormal production of the prototypical type 2 cytokines IL-4, IL-5, and IL-13 has long been connected with the pathogenesis of asthma.¹⁸ These interleukins that interceded with the significant pro-inflammatory role in asthma, as well as

induction of the IgE isotype switch, increased expression of vascular cell adhesion molecules, and stimulation of Th2 lymphocyte differentiation and mucus production, thus releasing IL-4, IL-5, and IL-13.¹⁹ Rats over-expressing IL-13 in the lungs exhibited numerous features of asthma such as airway epithelial hyperplasia, airway obstruction, pulmonary eosinophilia, and hyper-responsiveness to cholinergic stimulation. Even though both IL-4 and IL-13 induce an asthma-like phenotype, the activity of IL-13 appears to be superior to that of IL-4.²⁰ In the current study, 6-gingerol reduced the levels of IL-4, IL-5, and IL-13, consistent with levels of total IgE in serum.

Conversely, IFN- γ is a well-known Th1 effector and is associated with both chronic and acute stable asthma. It inhibits allergen-induced eosinophil conscription and IgE discharge via down-regulating IL-4 and IL-5 expression in the lung tissues of an asthma model.²¹ Earlier studies have demonstrated contradictory results that IFN- γ overexpression in

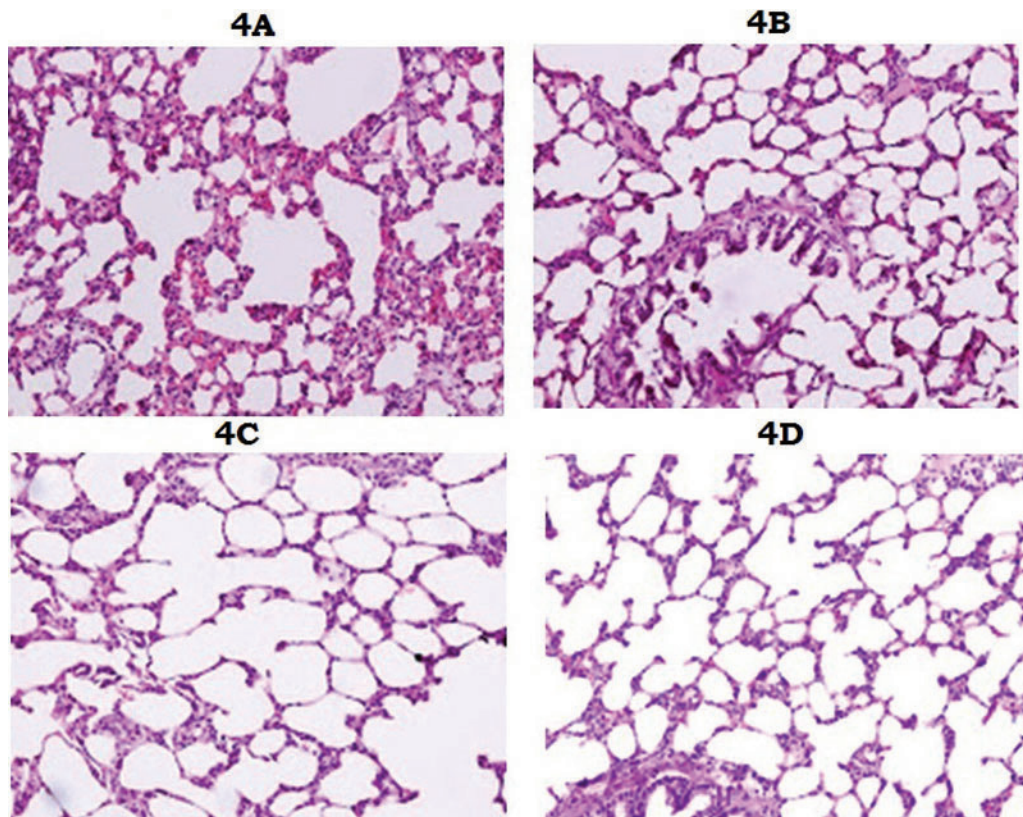


FIG. 4: Effect of 6-gingerol in lungs of control and experimental asthma rats. (A) Normal control: Normal control rats were showed normal histological architecture; H&E, 40 $\times$. (B) Asthma control: Asthma control rats showed that the glycoprotein compounds gathered in airways and all goblet cells in bronchioles were emptied and height of the bronchiolar epithelium was enlarged. (C) Asthma + 6-gingerol and (D) Asthma+ theophylline: Treatment with 6-gingerol and theophylline reduced most of the pathological alterations in OVA-induced asthma rats; H&E, 40 $\times$.

the airways also encourages noneosinophilic inflammation, lung fibrosis, and improved smooth muscle contractility.²² In this study, the IFN- γ level was notably decreased by OVA. Treatment with 6-gingerol or theophylline inhibited the production of inflammatory mediators IL-4, IL-5, and IL-13 and appreciably enhanced the reduced levels of serum IFN- γ due to the well-known immune suppression of 6-gingerol or theophylline.^{12,23} These findings proved that 6-gingerol or theophylline acted through altering both Th1 and Th2 cytokines responsible for eosinophil recruitment into lung tissue.

Histopathological modifications such as mucus hypersecretion, infiltration of inflammatory cells in lung tissue, and goblet cell hyperplasia are the most important features of asthma and are also loosely

linked to Th2 cytokines.²⁴ According to earlier findings,^{25,26} these biological cytokines play vital roles in directing β -cell growth, differentiation, and secretion of IgE and this prompts goblet cell hyperplasia and mucus hypersecretion. The action of IgE is due to its relations with F epsilon RI on mast cells and basophils and further cross-linking of F epsilon RI, thus initiating multiple signaling surges and leading to nuclear factor-kappa beta activation and the production of cytokines, lipid mediators, and chemokines.²⁷ Similarly, our present results found that serum levels of total IgE were significantly reduced by OVA-sensitized rats. Therefore, the inhibitory effect of 6-gingerol on elevated IgE levels appears to result from its suppression of Th2 cytokines, which are also known to induce airway hyper-responsive-

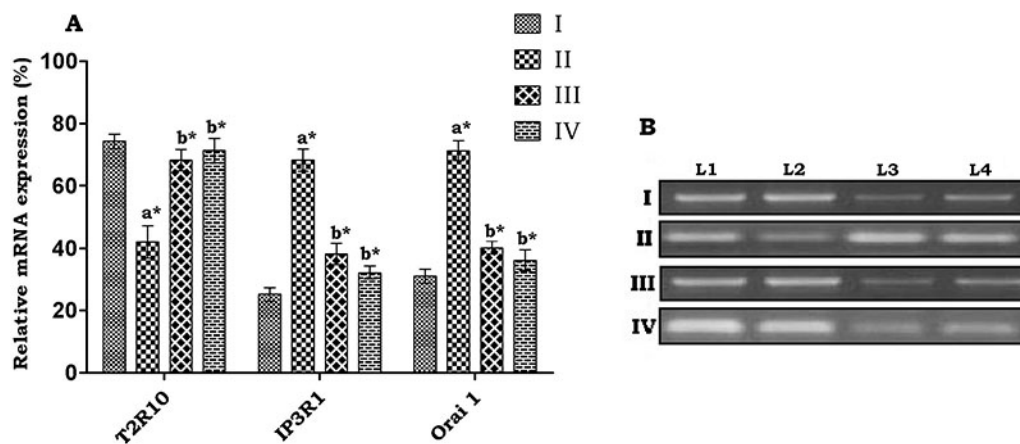


FIG. 5: Effect of 6-gingerol on mRNA expression of T2R10, IP3R1, and Orai 1 in control and experimental animals. L1: 18S; L2: T2R10; L3: IP3R1; L4: Orai 1, I: Normal control; II: Asthma control; III: Asthma + 6-gingerol; IV: Asthma + theophylline. Values are expressed as mean $\pm$ standard deviation for six animals in each group. Values are statistically significant at * $p < 0.05$. aSignificantly different from control. bSignificantly different from asthma control.

ness.²⁸ Airway hyper-responsiveness is the most important typical feature of asthma and the pathophysiologic corollary of the effect of inflammation progression.²⁹ In this study, we found that glycoprotein compounds deposit in airways, that all goblet cells in bronchioles were emptied, that the height of the bronchiolar epithelium was improved. Furthermore, the thickness of the epithelium in the alve-

oli was improved and many large nodules around bronchioles were observed in OVA-induced asthma and these alterations were restored after supplementation with 6-gingerol or theophylline. A probable justification for this result is that 6-gingerol could modulate the airway hyper-responsiveness via down-regulation of Th2 responses, thus accounting for its effect on asthma.

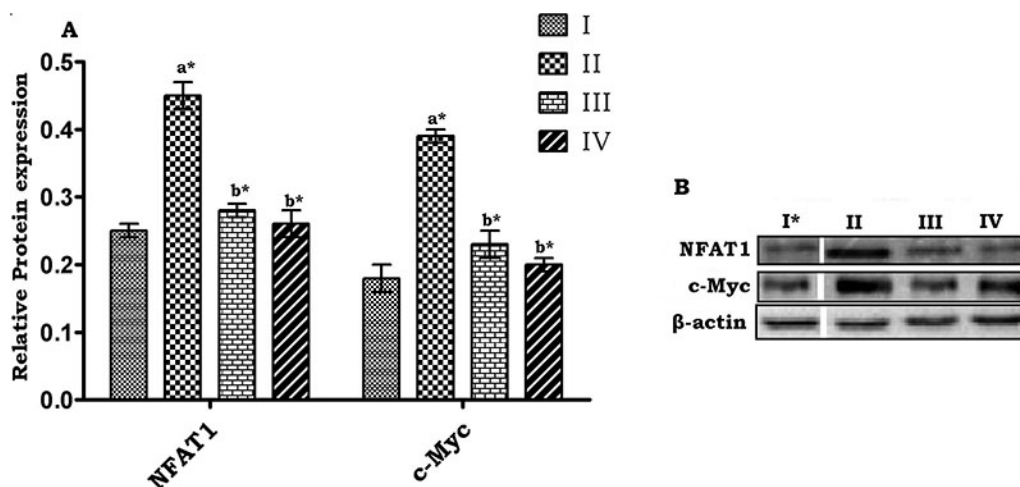


FIG. 6: Western blot analysis of NFAT1 and c-Myc protein expressions in control and experimental groups. I: Normal control; II: Asthma control; III: Asthma + 6-gingerol; IV: Asthma + theophylline. *Control blots were cropped from different part from the same gel. Values are expressed as mean $\pm$ standard deviation for six animals in each group. Values are statistically significant at * $p < 0.05$. aSignificantly different from control. bSignificantly different from asthma control.

T2Rs (bitter taste receptors), a subfamily of G-protein-coupled receptors, are one of the essential taste receptors on the tongue and defend against the consumption of potentially harmful substances, which are frequently bitter. T2Rs have also been recently established to be articulated in several cell types of the airways, including ciliated epithelial cells, solitary chemosensory cells, and smooth muscle cells of airways.³⁰ Bitter substances can activate T2Rs in airway smooth muscle (ASM) to source greater bronchodilation than β 2 adrenergic receptor agonists, the most regularly consumed bronchodilators. Mast cells and leukocytes from children with severe asthma have augmented the expression of T2Rs,³¹ whereas T2R agonists (such as chloroquine and denatonium) are the basis for dilation of airways and relaxation of isolated ASM.³² When asthma is treated with bronchoconstrictors, global intracellular content of Ca^{2+} ($[\text{Ca}^{2+}]_i$) is perceptibly augmented in T cells and ASM.³³ These Ca^{2+} changes in cells are arbitrated via activation of the canonical bitter taste signaling cascade (i.e., T2R-gustducin-phospholipase C β [PLC β]-inositol 1,4,5-triphosphate receptor [IP3R1]). Activation of T2Rs fully repeal the rise in $[\text{Ca}^{2+}]_i$ induced by bronchoconstrictors and this lessening of the $[\text{Ca}^{2+}]_i$ is essential for bitter tastant-induced ASM cell relaxation.³⁴

T2Rs may therefore be prospective targets for the improvement of a novel class of more effectual agonists for the treatment of asthma and bronchodilation.³⁵ In the present study, the anti-asthma mechanism of 6-gingerol was evaluated in an OVA-induced asthmatic rodent model. 6-Gingerol activated the expression of T2R10 in asthma rats. The results of RT-PCR analysis also demonstrated that 6-gingerol up-regulated T2R10 mRNA levels, but inhibited its key downstream targets (IP3R1) in the lung tissue of asthmatic rats. Furthermore, 6-gingerol could restrain the Orail mRNA expression, which regulates Ca^{2+} entry via governing Ca^{2+} release-activated Ca^{2+} channel.³⁶ Treatment with 6-gingerol down-regulated the protein levels of c-Myc and NFAT1. As the most downstream effectors in the T2R pathway, the transcription factor c-Myc and NFAT1 collaborates both with the mitogen activated protein kinase/Ras pathway to encourage inflammation and cell death in different cell types such as fibroblasts, Burkitt's lymphoma cells, and T lymphocytes.³⁷ These

results are consistent with the inhibition of 6-gingerol on inflammatory cytokine (IL-4, IL-5, and IL-13) production and suggest that 6-gingerol treatment can revolutionize OVA-induced inflammation via T2R10/IP3R1/NFAT1 signaling pathway.

V. CONCLUSIONS

In conclusion, this study revealed that 6-gingerol inhibits airway inflammation, ameliorates airway hyper-responsiveness, and suppresses Th2 cytokine and IgE levels by regulating the T2R10/IP3R1/NFAT1 pathway. The results of this research also showed that 6-gingerol restores histopathological alterations of the lung in asthmatic rats. These findings support a role of 6-gingerol as part of a dietary intake treatment for asthma and airway inflammatory disease.

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