# Inhibitory Effect of E-RH-06 on HDM-induced Secretion of IL-6 and IL-8 in rhinitis

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***Abstract-*** *Background:* Rhinitis, an extremely common medical condition, is characterized by acute or chronic inflammation of mucous membranes. Pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-8 and TNF-α are the key drivers of inflammatory response in rhinitis. Hence, their inhibition is an important therapeutic strategy for treating rhinitis. In vitro cell line models are extremely valuable for testing the potential of new therapeutic compounds. Epithelial cell lines derived from the airway and nasal passage have been previously reported as models for testing anti-rhinitic drugs such as Cetirizine and Loratidine.

*Objectives:* This study aimed to test the anti-rhinitic activity of the test compound E-RH-06 using A549 human airway epithelial cells. A549 cells were pre-treated with non-cytotoxic concentrations of E-RH-06 for 24 hours before stimulation with human dust mite (HDM) allergen. Anti-rhinitic potential was evaluated by quantitation of IL-6, IL-8 and TNF-α pro-inflammatory cytokine levels secreted by cells into the extracellular culture medium.

*Results:* Treating A549 cells with the inflammatory stimulus, HDM extract, resulted in an overall increase in IL-6 and IL-8 levels, thus, stimulating inflammation, as prevalent in rhinitis. However, no considerable levels of TNF- α were detected. Pre-treatment of A549 cells with E-RH-06 resulted in inhibition of IL-6 and IL-8 cytokine levels compared to control cells ($p<0.001$). A similar level of inhibition was observed with the loratidine positive control treated cells.

*Conclusions:* The study demonstrate that E-RH-06 possesses anti-rhinitic activity, described as inhibition of HDM-induced secretion of pro-inflammatory cytokines, warranting further investigation of its potential as a novel anti-rhinitic therapeutic compound.

***Index Terms***- rhinitis, anti-rhinitic potential, inflammatory stimulus, human epithelial cell, human dust mite allergen

## I. INTRODUCTION

Rhinitis, including hay fever or seasonal allergic rhinitis, is an extremely common condition which is increasing around the world. Rhinitis rates vary largely across the world, with prevalence rates for adults aged 25-50 years estimated at 12% in Spain, 30% in the United States and 46% in Australia. Consequently, in many countries, rhinitis poses a substantial healthcare burden both in terms of treatment costs, indirect costs and quality of life and associated comorbidities. As such, the development of new and improved therapies to improve the quality of life and reduce the social and economic burden of rhinitis is a constant target [i,ii]

Rhinitis is a medical term describing irritation and inflammation of the mucous membrane of the nasal passage which may be caused by viruses, bacteria, allergen or irritants[iii]. Acute or chronic inflammation of the mucous membrane, a hallmark of rhinitis, results in the generation of excessive amounts of mucus, runny nose as well as nasal congestion, sneezing and itchiness of nose, throat, eyes and ears, causing considerable to discomfort to the individual. The allergic rhinitis is usually triggered by airborne allergens such as pollen and mold spores[iv]. Indoor allergens such as animal dander or dust mites are common causes of year-round allergic rhinitis[v]. Additional symptoms of allergic rhinitis are sneezing, nasal itching, coughing, headache, fatigue, malaise, and cognitive impairment. The allergens may also cause watery, reddened or itchy eyes and puffiness around the eyes[vi].

The characteristic inflammatory response observed in rhinitis is initiated by antigen-presenting cells (APC), which are primed following recognition of a 'foreign' agonist such as the house dust mite (HDM) allergen. APC-priming results in activation of a T helper type 2 (Th 2) cell-mediated immune response, which involved a number of key pro-inflammatory cytokines including TNF-α, IL-6, IL-8, IL-4, IL-5, IL-13, and GMCSF, and chemokines such as eotaxin & RANTES that play pivotal roles in the development of the inflammatory milieu in rhinitis[vii,viii]. These biomarkers significantly contribute to the inflammation of nasal airway passages and promote the progression of rhinitic conditions[ix,x]. Upon treatment with external allergens or stimulating agents, these cytokines show variation in their secretory levels. Assessing these modulations in the secretion of the cytokines provides important insights on the anti-rhinitic activity of many potential drugs[xi].

In vitro stimulation of cells with rhinitis causing agents, such as HDM extract promotes the excessive secretion of pro-inflammatory cytokines & chemokines and stimulates the inflammatory environment prevalent in rhinitis. Airway epithelial cells (A549) have been reported as a cell model to investigate stimulation of pro-inflammatory cytokines such as IL-6 & IL-8, upon treatment with HDM extract[xii]. These cells have also been reported as a model to evaluate inhibition of pro-inflammatory cytokines such as IL-8, by anti-rhinitic drugs such as Cetirizine and Loratadine[xiii]. In addition, stimulation of the nasal epithelial cell line RPMI 2650 with HDM extract is reported to mimic the inflammatory scenario prevalent in rhinitic conditions[xiv]. Therefore, airway epithelial cells and nasal epithelial cells may be valuable therapeutic targets for discovery

and development of new drugs or therapeutic strategies to treat rhinitis [xv,xvi].

The aim of this study was to investigate the anti-rhinitic activity of a new test compound, E-RH-06, using a cell line which has previously been shown to have major involvement in the pathogenesis of rhinitis: A549 human airway epithelial cells. Anti-rhinitic potential of E-RH-06 was evaluated by assessing the expression of the inflammatory cytokines TNF-α, IL-6 & IL-8.

## II. MATERIALS AND METHODS

*Reagents*

The test compound E-RH-06, a new product of Enovate Biolife Pvt. Ltd. (Mumbai, India), is a premix in powder form, grayish brown in color. It was maintained as a 100 mg/ml stock solution in DMSO. Working solutions for cell treatment in the range of 0.1 µg/ml - 500 µg/ml were made by further dilution of the stock solution with serum free medium. The positive control compound, Loratadine (Morepore Laboratories Limited, India), was dissolved in DMSO to obtain a 1 mg/ml (2.6 mM) stock solution. This stock solution was further diluted in serum free medium to prepare further dilutions for cell treatment in the range of 0.00038 µg/ml-3.8 µg/ml.

*Cell line*

The human airway epithelial cell line, A549, was purchased from the National Centre for Cell Science (Pune, India) and grown in Ham's F12 media (Himedia) supplemented with 10% FBS at 37°, 95% humidity and 5% $CO_2$.

*Determination of non-cytotoxic concentrations of E-RH-06*

A549 cells were seeded at a density of 5000 cells / well in 96-well culture plates and incubated at 37°C, 95% humidity and 5% $CO_2$ overnight. Cells were treated then with E-RH-06 in serum free medium corresponding to final concentrations ranging from 0.1 µg/ml-500 µg/ml in triplicates. Cells treated with DMSO (0.5%) were used as controls.

Cell viability was determined 48 h after E-RH-06 treatment, by the addition of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml). After 3 h, supernatants were removed; the formazan complex formed was resuspended in 150 µl of DMSO and absorbance of the samples measured at 540 nm.

The percentage cytotoxicity of E-RH-06 at respective concentrations was calculated using the following formula: Cytotoxicity percentage = [(Y-X)/Y]* 100, where, X = Absorbance of cells treated with E-RH-06 at 540 nm and Y = Absorbance of cells treated with 0.5% DMSO at 540 nm. E-RH-60 concentrations, which resulted in >80% viability of cells, were determined and selected for subsequent cytokine analysis studies.

*Evaluation of anti-rhinitic potential of E-RH-06*
*HDM extract induced rhinitic assay*

A549 cells were seeded in triplicates at a density of $0.1x10^6$ cells/ml/well in 24-well culture plates and incubated at 37°C, 95% humidity and 5% $CO_2$ overnight. Cells were treated with E-RH-06 in serum free medium at non-cytotoxic concentrations (0.1 µg/ml - 100 µg/ml) for 24 h. Subsequent to treatment with E-RH-06, cells were stimulated using house dust mite (HDM; Creative Diagnostic Medicare Pvt. Ltd, India) extract (500 U/ml) for 24 h. Cell culture supernatants were collected from each well and stored at −20 °C for cytokine analysis. Cells treated with vehicle and HDM extract were used as controls. Cells treated with Loratadine and HDM extract were used as positive controls.

*Estimation of cytokines using ELISA*

Levels of TNF-α, IL-6 & IL-8 were estimated at five non-cytotoxic concentrations of E-RH-06, selected on the basis of the cytotoxicity assay, using an Enzyme Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions (R&D Systems Inc, MN, USA). These assays employ the quantitative sandwich enzyme immunoassay technique. Commercially available polyclonal antibody, specific for mouse cytokine (pre-coated onto a microplate) was used. Standards, controls, and test samples (200 µl for TNF-α. 100 µl for IL-6 and 50 µl for IL-8) were added into the wells. Mouse antigen present in either of the samples was bound by the immobilized antibody. After washing any unbound substances away, an enzyme-linked polyclonal antibody, specific for cytokine/chemokine, was added to the wells (200 µ1 for TNF-α, IL-6 and 100 µl for IL-8). Following removal of any unbound antibody-enzyme reagent by washing, 200 µl of substrate solution was added to the wells. After 30 min, 50 µl of stop solution was added. The intensity of color was measured at 450 nm and was proportional to the amount of cytokine/chemokine bound in the initial step. Concentrations of cytokines were then determined from the standard curve. Percentage change in the levels of secreted cytokine in culture supernatants was determined using the following formula: Change in cytokine level = [(B-A)/B]*100, where A = Concentration of cytokine (pg/ml) secreted by HDM extract-stimulated cells pre-treated with E-RH-06 and B = Concentration of cytokine (pg/ml) secreted by HDM extract + vehicle stimulated cells.

*Statistical Analysis*

Data was analysed using Graph Pad PrismTM software v4.01. Differences in cytokine expression in treated, and control cells, were analysed using one-way ANOVA (Bonferroni post test) for test of significance. P values <0.05 were considered significant.

## III. RESULTS

*Identification of non-cytotoxic concentrations of E-RH-06*
*Effect of E-RH-06 on the viability of A549 cells*

To determine the effect of E-RH-06 on the viability of A549 cells, cells were treated with a concentration range of 0.1 µg/ml – 500 µg/ml for 48 h. Compared to the vehicle control more than 80% viability was observed at the following concentrations: 0.1 µg/ml, 10 µg/ml, 50 µg/ml & 100 µg/ml (Figure 1). These concentrations were, therefore, selected for cytokine analysis.

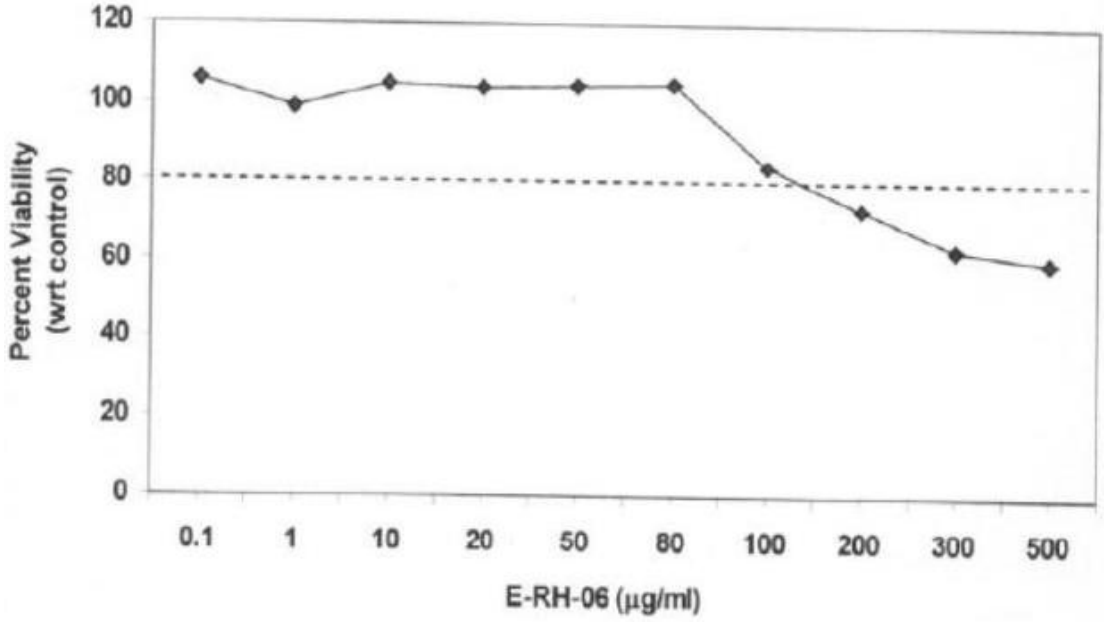


**Figure 1: Effect of E-RH-06 on viability of A549 cells after 48 h of treatment.** *Each point represents mean value of triplicates

No loss of viability in A549 cells was observed with Loratadine treatment in the concentration range of 0.00038 µg/ml to 3.8 µg/ml (Figure 2).

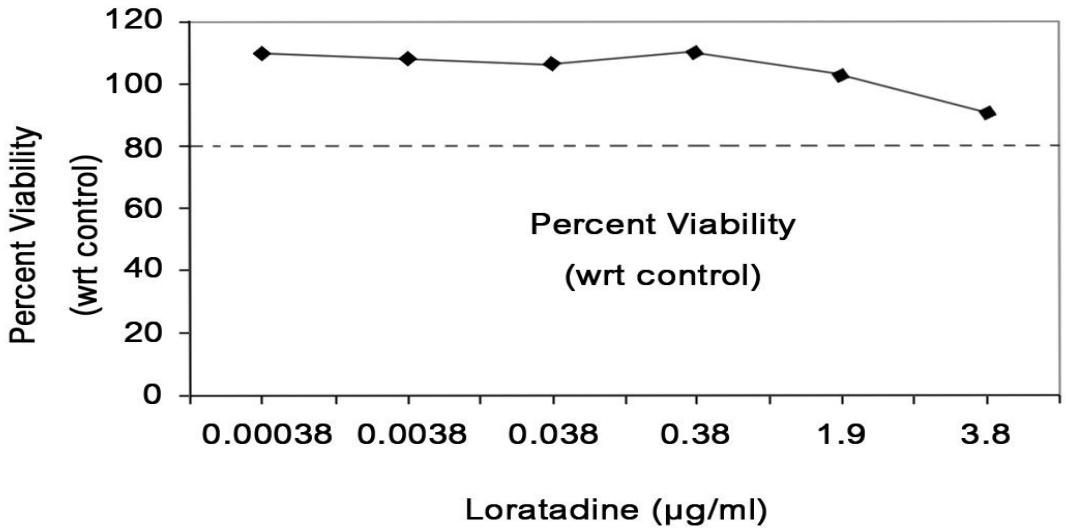


**Figure 2: Effect of Loratadine on viability of A549 cells after 48 h of treatment.** *Each point represents mean value of triplicates.

*Estimation of cytokine activity*

*Effect of E-RH-06 on expression levels of cytokines secreted by A549 cells*

Treatment of A549 cells with HDM resulted in stimulation of cytokine secretion into the culture supernatants. The expression levels of IL-6, IL-8 and TNF-α were estimated in culture supernatants of A549 cells pretreated with E-RH-06 and stimulated with HDM extract in order to evaluate the anti-rhinitic activity of E-RH-06. Cytokine estimation was performed at the following non-cytotoxic concentrations of E-RH-06 in triplicates: 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml.

Secreted levels of IL-6 in A549 cells stimulated with HDM-extract is shown in Figure 3. Pretreatment of A549 cells with 0.1 µg/ml, 1 µg/ml and 10 µg/ml E-RH-06 for 24 h resulted in inhibition of IL-6 secretion into the extracellular medium by 46 %, 51.3 % and 43.4 % respectively as compared to control cells (HDM extract + vehicle; $p<0.001$). Down-regulation of IL-6 levels was also observed in cells treated with the loratadine

(0.038 to 0.38 µg/ml) positive control. IL-6 levels were increased at high concentrations of E-RH-06 (50 µg/ml and 100 µg/ml).

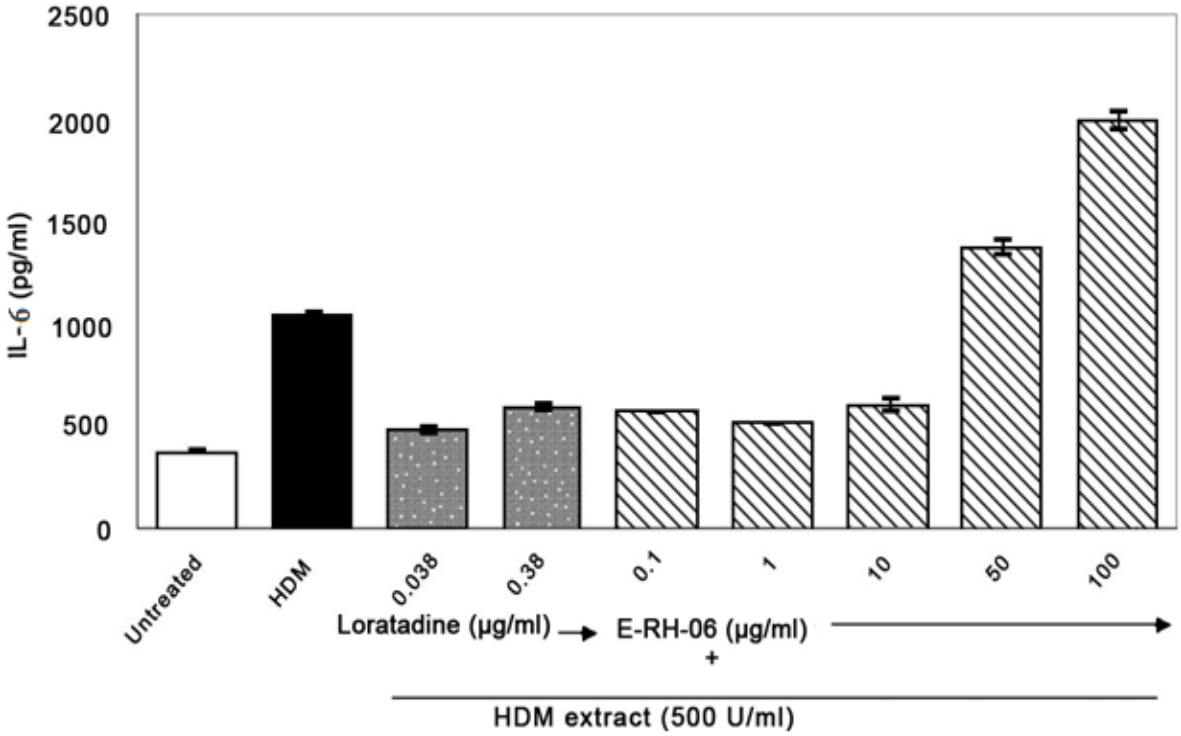


**Figure 3: Effect of E-RH-06 on the expression levels of IL-6 secreted by HDM extract-stimulated A549 cells.** *Each point represents mean value and S.E. of triplicates. A significant decrease ($p<0.001$) in IL-6 levels was observed at 0.1, 1 and 10 µg/ml of E-RH-06 as analyzed by One-way ANOVA (Bonferroni post test).

Pretreatment of A549 cells with E-RH-06 prior to HDM-extract stimulation also resulted in inhibition of IL-8 activity (Figure 4). A reduction of 50.3 % - 66.6 % in IL-8 secretion by A549 cells was observed in cells pretreated with E-RH-06 in the concentration range of 0.1 µg/ml – 100 µg/ml as compared to control (HDM extract + vehicle) cells ($p<0.001$). Down-regulation of IL-8 was also observed in the loratadine (0.038 to 0.38 µg/ml) positive control treated cells.

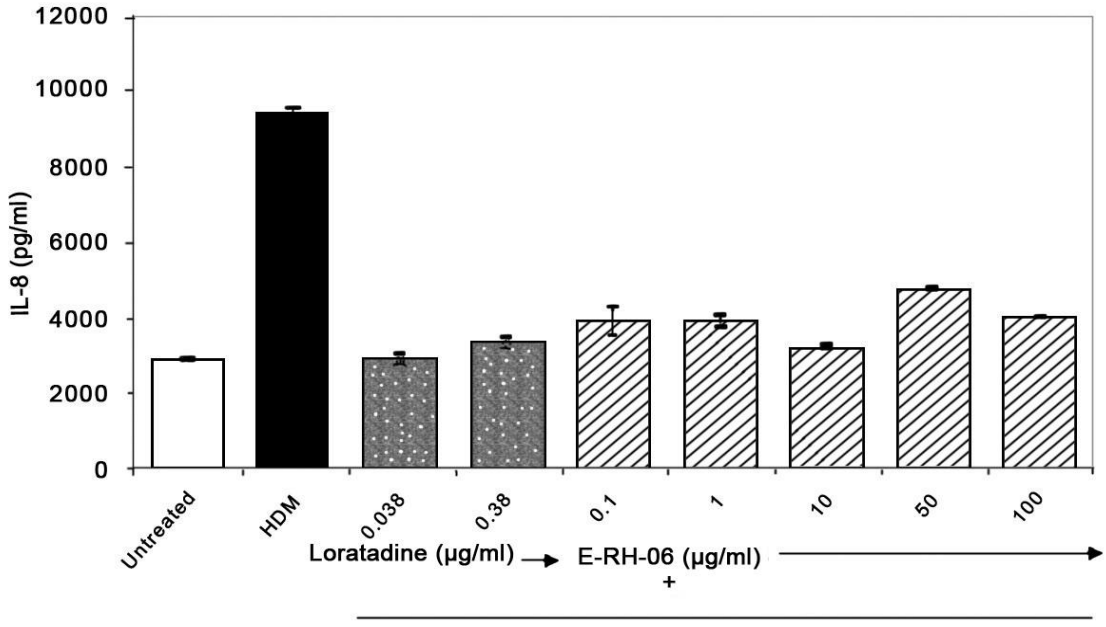

**Figure 4: Effect of E-RH-06 on the expression levels of IL-8 secreted by HDM extract-stimulated A549 cells**. *Each point represents mean value and S.E. of triplicates. A significant decrease ($p<0.001$) in IL-8 levels was observed at 0.1 µg/ml – 100 µg/ml of E-RH-06 as analysed by One-way ANOVA (Bonferroni post test).

The levels of TNF-α were undetectable in culture supernatants of A549 cells treated with vehicle such as HDM extract or E-RH-06 + HDM extract.

## IV. DISCUSSION

Rhinitis is an increasingly common medical condition which is associated with inflammation of the nasal and airway epithelium leading to irritation[xvii]. Inflammation is mediated through expression of pro-inflammatory cytokines, including IL-6, IL-8 and TNF α, in response to stimulation by foreign agonists such as allergens, irritants, bacteria and viruses[xviii,xix]. Many current anti-rhinitic therapies aim to reduce the levels of such cytokines in order to down-regulate the inflammatory response and associated irritatory symptoms [xx,xxi,xxii].

Rhinitis may be mimicked in vitro through stimulation of the cell line models, for example, the nasal epithelial RPMI 2650 cell line and airway epithelial A549 cell line, with allergens such as human dust mite extract[xxiii] . In such model systems, inflammatory stimulation results in extracellular secretion of pro-inflammatory cytokines, providing a direct measure of inflammatory response and enabling the anti-inflammatory potential of therapeutic compounds to be simply evaluated[xxiv,xxv]. In the present study, we elucidated the anti-rhinitic potential of a new compound, E-RH-06, using a human airway epithelial cell line (A549) by assessment of modulation of the secretion of key pro-inflammatory cytokines against HDM extract induced inflammation.

Treatment of A549 cells with E-RH-06 at non-cytotoxic concentrations (0.1 µg/ml-100 µg/ml) resulted in inhibition of HDM extract-induced levels of IL-6 by 43% - 51.3%. Similarly, IL-8 levels were reduced by 50.3-66.6%, following pre-treatment with E-RH-06 (0.1 µg/ml-100 µg/ml). IL-6 and IL-8 are key pro-inflammatory cytokines which mediate production of other effector molecules of the cytokine network in inflammation. Hence, inhibition of these cytokines should down-regulate the inflammatory milieu associated with rhinitis.

A limitation of this study was the use of only a single cell line for the evaluation of anti-rhinitic activity of E-RH-06. In addition, as TNF-α level could not be detected in the extracellular medium of A549 cells. The results of this study may be further corroborated through the use of a different cell line or through examination of additional cytokines in the A549 cell line.

In summary, the data obtained from the present study indicates the potential anti-rhinitic activity of E-RH-06. Pre-treatment with E-RH-06 was associated with considerable down-regulation of secreted pro-inflammatory cytokines IL-6 & IL-8 into the microenvironment of A549 cells in response to HDM extract induced inflammation. The ability of E-RH-06 to inhibit IL-6 and IL-8 suggests potential as a therapy to reduce the inflammatory cascade associated with rhinitis, and therefore, relieve the associated symptoms which cause considerable discomfort to individuals. Further investigation is, therefore, warranted to confirm the anti-rhinitic activity of E-RH-06.

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