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Research Article

A Comparative Evaluation of UNIM-352, a Unani Polyherbal Preparation, and its Optimized Formulations, on Oxidative Stress and Bronchial Hyperresponsiveness in Experimental Model of Bronchial Asthma

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Abstract

UNIM-352 is a polyherbal agent used in Indian Unani traditional system of medicine for bronchial asthma containing a combination of the following well-known medicinal plants: *Linum usitatissimum, Trigonella-foenum-graecum, Allium sativum, Strychnos potatorum, Bonducella fleming and Pongamia glabra, Honey (q.s.)*. However, this effective and validated formulation can achieve better global acceptance only if the number of ingredients is reduced without affecting its efficacy and safety. In the present study, an attempt has been made to optimize UNIM-352 by comparing the efficacy and safety of the conventional agent with four different optimized preparations (OP) containing 3 - 4 ingredients on oxidative stress and bronchial hyper-responsiveness in experimental models of asthma. Rats (n = 6/group) were immunized and challenged with OVA for developing the allergic asthma model and blood and BAL fluid samples were assayed for oxidative stress markers. UNIM-352 and optimized preparations of UNIM-352 (OPs) showed differential degree of attenuations on oxidative stress markers; viz. OP-1 and OP-3 were more effective in reducing MDA and OP-2 and OP-4 induced greater reductions in NOx levels as compared to experimental control group of rats. On the other hand, comparable augmentations in the levels of antioxidants, GSH and SOD, were seen with optimized preparations OP-3 and OP-4and UNIM-352. In studies of bronchial hyper-responsiveness, *P-enh*, an indicator of bronchial hyperreactivity and resistance to airflow, was increased in control animals (OVA Immunized + methacholine challenged), which was reduced after treatment with UNIM-352 and the optimized preparations of UNIM-352 viz. OP-1, OP-3 and OP-4. The study shows that optimized preparations of UNIM-352, OP-1 and OP-3 were more effective as compared to the conventional UNIM-352 preparation in modulation oxidative stress markers and bronchial hyperreactivity. These optimized (OP) UNIM-352 preparations, with reduce number of ingredients, could thus be used as effective and better alternatives to the conventional Unani polyherbal formulation and enhance the global acceptability of this unique anti-asthma drug.

Keywords: *UNIM-352; Optimized Formulations; Ovalbumin; Asthma; Bronchial Hyper-Responsiveness; Oxidative Stress*

Introduction

Respiratory disorders are one of the leading causes of mortality and morbidity and are major global health problem. The overall worldwide prevalence of bronchial asthma is approximately 4.5 percent. About 334 million patients have been affected globally with asthma across all age groups. The disease prevalence of bronchial asthma has increased with time and an approximately 100 million more people globally are at a risk

to develop asthma by the year 2025 [1]. Bronchial asthma is characterized by chronic airway inflammation and hyper-responsiveness along with reversible airway obstruction. It is diagnosed by the history of respiratory symptoms like wheezing, shortness of breath, cough, chest tightness that varies over intensity and in time, accompanied with inconsistent expiratory airflow obstructions. Variations in expiratory airflow obstructions are triggered by different stimuli, including allergen exposure, exercise, viral-infection (cold) and respiratory airflow irritants like changes in weather, smoke and vehicle exhaust fumes [2]. Airway inflammation and hyper-responsiveness are the main distinctive features of bronchial asthma. In atopic individuals, specific antigen re-exposure causes an instant early-phase response. Persistent inflammation in asthma may lead to structural changes in airway lumen and other components of the airway termed airway remodeling. Changes in airway remodeling consists of sub-epithelial fibrosis, airway wall thickening, and hyperplasia of myofibroblasts, goblet cell and smooth muscle [3]. This is followed by late phase response, after 6 - 12 hours, consisting of mast cell degranulation mediated by IgE and production of inflammatory mediators like histamine and is primarily linked with recruitment of eosinophils [4-6].

In most asthmatic patients, relapse occurred after withdrawal of therapy. Inhaled corticosteroids are usually efficacious but long term use is associated with both systemic and local adverse effects viz. osteoporosis, immune suppression and increased susceptibility to infections, cataract in elderly patients, and behavioral changes. Long term use of beta agonists is also associated with debilitating tremors and reduced bronchodilator responses - due to beta receptor sub-sensitivity. Taken together, all these issues give rise to refractoriness to treatment, reduced compliance and increased morbidity and mortality [7].

Thus, there is a constant search for viable complimentary/alternative forms of pharmacotherapy to improve the treatment outcome and quality of life in asthma. In the Indian traditional systems of medicine, plant derived products play a key role and constitutes a major source for therapeutic agents. Validation studies of various medicinal plants species-alone and in combination, have demonstrated the efficacy of plants in reducing respiratory symptoms [8,9]. Complementary and alternative systems of medicine e.g. Ayurveda and Unani, use plant derived agents for therapeutics. These traditional systems of medicine have been generally accepted in most of the countries and referred as Complementary and Alternative Medicine (CAM) [11]. The Golden triangle project initiated by the Government of India (involving ICMR, CSIR and AYUSH) has given further impetus to such efforts. Since ancient times, various plants and plant derived products have been used traditionally for the treatment of bronchial asthma throughout the world and have been reported in literature and also scientifically proven to have anti-asthmatic activity. UNIM-352 is a formulation containing a combination of the following well-known medicinal plants: *Linum usitatissimum L*. (1g), *Trigonella-foenum-graecum L*. (2g), *Allium sativum L*. (3.5g), *Strychnos potatorum L*. (7g), *Caesalpinia bonducella* Fleming (1g), *Pongamia glabra Vent* (1g), and Honey (q.s.) in 20g of preparation. This polyherbal traditional therapy has been proven efficacious in the management of asthmatic patients, and its cellular and molecular mechanisms of action has been proposed [12,13]. A pilot clinical study with the formulation has shown that UNIM-352 can be used as an adjuvant treatment of bronchial asthma as it increased the efficacy of conventional drugs by way of improving the lung functions and reducing the incidences of acute exacerbations of the disease [14].

UNIM-352 was shown to be safe and effective in clinical and experimental studies, and it was decided to reduce the number of botanical components in the formulation in order to facilitate its global acceptability without compromising on the efficacy. It is worth mentioning here that in countries of Europe and in the US optimal number of ingredients (usually 3 or 4) is preferred [8]. So, this study was designed to down size the number of ingredients in UNIM-352 and validate their effects in experimental models of asthma without affecting its efficacy and/or safety. Therefore, an attempt has been made to optimize UNIM-352 by comparing the efficacy and safety of the original formulation constituted of six ingredients with the new optimized preparations (OP) with successive elimination of 1 to 3 ingredients, which were deemed less important in light of available scientific literature. The study also evaluated the possible molecular mechanisms of actions of optimized preparation (OP) of UNIM-352 in experimental models of bronchial asthma in rats [15]. The effects of optimized preparations of UNIM-352 were also assessed on the markers of oxidative stress as there is evidence of dysregulation of redox balance in asthma and both reactive oxygen and nitrogen species have been implicated [16]. Additionally, effects of optimized preparations of UNIM-352 were assessed on airway hyperresponsiveness and airway resistance in conscious rats by whole body plethysmography.

Materials and Methods

Animals

In this study, Wistar rats (180 - 250g) were used. Animals were kept in polyacrylic cages in standard controlled room temperature (22 ± 2ºC) with natural light and dark cycle. Rats were fed with food pellets and water ad libitum. The protocol was approved by Institutional Animal Ethics Committee (IAEC/7/2015) following the guidelines of CPCSEA; Animal care was as per the guidelines of Indian National Science Academy (INSA), New Delhi.

Drugs and chemicals

UNIM-352 and various optimized preparations (OP) of UNIM-352 were used in our study. The formulations were prepared, standardized and supplied by Central Research Institute of Unani Medicine (CRIUM) Hyderabad, under the auspices of Central Council for Research in Unani Medicine (CCRUM) Ministry of AYUSH, Govt of India, and the contents certified.

UNIM-352 classically contains six constituents viz. *Linum usitatissimum L* (1g), *Trigonella foenum-graecum L* (2g), *Allium sativum L* (3.5g), *Strychnos potatorum L* (7g), *Caesalpinia bonducella Fleming* (1g), *Pongamia glabra Vent* (1g) and Honey (q.s.) in 20g of preparation.

Optimized preparation-1 (OP-1) contained 3 ingredients*: Strychnos potatorum L, Trigonella-foenum-graecum L, Pongamia glabra Vent* and Honey.

Optimized preparation-2 (OP-2) contained 4 ingredients*: Strychnos potatorum L, Trigonella-foenum-graecum L, Pongamia glabra Vent, Allium sativum L*, and Honey.

Optimized preparation-3 (OP-3) contained 4 ingredients*: Strychnos potatorum L, Trigonella-foenum-graecum L, Pongamia glabra Vent, Linum usitatissimum L*, and Honey.

Optimized preparation-4 (OP-4) contained 4 ingredients*: Strychnos potatorum L, Trigonella-foenum-graecum L, Pongamia glabra Vent, Caesalpinia bonducella Fleming*, and Honey.

Chemicals

Ovalbumin, Methacholine, and Prednisolone were purchased from M/s Sigma-Aldrich, St Louis, MO, USA. Aluminum hydroxide, Sodium chloride, Hydrochloric acid, Sodium hydroxide, propanol, Perchloric acid, Glacial acetic acid, Formaldehyde, and other chemicals used in our study were purchased from Central Drug House (P) Ltd, New Delhi.

Immunization and challenge protocol

All rats in each group except normal group were immunized and challenged with OVA according to the procedure mentioned by Kwasniewski., *et al* [17]. Briefly, rats were sensitized by intraperitoneal injection of OVA (10 mg per rat) emulsified with 10 µg of aluminum hydroxide in 0.5 ml of normal saline on the first day of the study. Fourteen days after sensitization, animals were challenged with 1 mg of ovalbumin.

Blood and bronchoalveolar lavage fluid collection (BAL) and cell counts

After 24 hrs of OVA challenge, all animals were anesthetized with ketamine dose 50 mg/kg and blood collected by cardiac puncture, the sample were centrifuged at 3000 rpm for 10 min at 4ºC. Serum was separated and stored at -80ºC. BAL fluid was retrieved by washing the lung

through tracheal cannula with 0.9% sodium chloride solution and centrifuged at 1500 rpm at 4ºC for 10 min. The supernatant recovered was stored at -80ºC for the assay of various cytokine. The precipitated pellets were resuspended in 100 µl of normal saline. Eosinophil and Neutrophil counts in blood and BAL fluid were carried out using Neubauer chamber after Leishman staining [18].

Assays for estimation of oxidative and nitrosative stress markers

Assay for MDA levels [19]

In this assay Malondialdehyde (MDA) was determined, as a marker of lipid peroxidation. The reaction mixture consisted of 0.2 ml of 8.1% of sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% of aqueous solution of thiobarbituric acid and 0.2 ml of serum sample. The mixture was then made up to 4 ml using distilled water heated for 60 min at 95ºC. After cooling with tap water, 5 ml of n-butanol and pyridine in the ratio of (15:1) and 1 ml of distilled water was added and centrifuged. After the organic layer was separated its absorbance was measured using spectrophotometer at a wavelength of 532 nm and results were expressed as nM/mg of protein.

Assay for superoxide dismutase (SOD) levels

Superoxide dismutase (SOD) which catalyzes the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide is among the most important anti-oxidative enzyme. The assay of SOD was performed by the method devised by Nandi and Chatterjee [20]. Assay mixture containing 2.86 ml of trisHCl buffer (50 mM, pH-8.5) and 0.1 ml EDTA (30 mM) was added to 20 µl serum or BAL fluid sample. After that 20 µl of 2.6 mM pyrogallol was added and immediately absorbance was taken at 420 nm for two minutes at 30 second interval for calculating the units of Superoxide dismutase (SOD) activity in the samples. The SOD activity was expressed as U/mg protein.

Assay for reduced glutathione (GSH)

Serum glutathione (GSH) levels were estimated by the method of Ellman [21]. The assay is based on the procedure of enzymatic recycling in which glutathione is sequentially oxidized by the DTNB and reduced by NADPH in presence of glutathione reductase. 0.1 ml of serum was mixed with 2 ml of phosphate buffer and 0.5 ml of DTNB. Further 0.4 ml distilled water added and the whole reaction mixture was vortexed and the level of 2-nitro-5-benzoic acid formation was monitored as an increase in absorbance at 412 nm and results are expressed in µg/mg protein.

NO metabolite (NOx) assay

As the half-life of NO is few seconds, the stable metabolites of nitric oxide viz. total nitrites and nitrates (NOx) were estimated by the spectrophotometric method of Tracey., *et al* [22]. In this method, aspergillus nitrate reductase was coupled with FAD and NADPH to convert all nitrates present within the sample to nitrites. The assay mixture contained 20 µl plasma, 10 µl of 0.86 mM/ml NADPH, 10 µl of 0.11 mM/ml FAD, 10 µl of nitrate reductase (2 U/ml) and 50 µl of 310 mM/ml of potassium phosphate buffer (pH-7.5) in the total assay volume of 100 µl. Samples were then incubated for 1 hour at 37ºC in dark followed by the addition of 5 µl of 1 mM/ml potassium ferricyanide and 5 µl of 1 M/ml zinc sulphate to oxidize the unreacted NADPH and precipitate the proteins respectively. After centrifugation of microtubes containing supernatant, 50 µl from each microtube was transferred into every well of 96 well microplate which was followed by addition of 100 µl Griess reagent [1:1 mixture of 1% sulfanilamide in 5% ortho-phosphoric acid and 0.1% N-1-napthyl] ethylene diamine for colour development. Readings were measured in a 96 well assay plate after 10 min at 540nm using the software-based microplate reader (ECIL). Standard curve was generated with known concentration of sodium nitrate and results were expressed in nmoles/mg of protein.

Measurement of bronchial responsiveness to spasmogen

In the experiment model of bronchial asthma, after 24h of last challenge of OVA, airway responsiveness was assessed in response to methacholine inhalation using whole body plethysmography (WBP) and expressed as enhanced pause (*P-enh*) [23]. Whole body plethysmography is a non-invasive technique that measures lung function by enhanced pause (*P-enh*). The non-invasive Penh method is suitable for repeatable and quick screening of respiratory function in large number of conscious rats in normal breathing conditions. Airway hyperresponsiveness (AHR) is a typical end point to assess lung function in rats sensitized and challenged with ovalbumin. AHR is mostly tested using inhalational provocation with increasing doses of the bronchoconstrictor agent, methacholine. WBP has some advantages compared to invasive means for the measurement of lung resistance. Since WBP is technically less demanding and also allows measurements of airway responsiveness to aerosolized stimulants. Briefly, rats were placed in a whole-body plethysmography chamber. *P-enh* reading were taken for average period of 3 minute. Subsequently, the rats were exposed to increasing dose of aerosolized methacholine (2.5, 5 and10 mg/ml), for 3 min and then the reading of *P-enh* were taken for an average of 3 minutes after each dose nebulization [24]. Changes in the lung volume during compression and decompression of thoracic gas were calculated while the animals breathed completely with plethysmograph. The fundamental principle of the plethysmograph is that change in the alveolar pressure is inferred from changes in plethysmograph pressure [25]. *P-enh* an empirical parameter evaluates changes in the box flow from both expiration and inspiration and then combines it with the comparison of early and late expiratory phase and used to measure airway resistance which is increased during bronchoconstriction.

Statistical analysis

The data was expressed as mean ± S.E.M. Statistical analysis was done by using one-way analysis of variance (ANOVA) followed by post hoc Tukey`s test for intergroup comparisons. A p value of at least 0.05 was used as the level of significance in all statistical tests.

Results

Effects of UNIM-352 and optimized preparations of UNIM-352 on oxidative and nitrosative stress markers in blood in OVA sensitized and challenged rats

Oxidative stress is defined as increase in oxidative source (pro-oxidant) and reduced anti-oxidant defense mechanisms which play a significant role in inflammatory diseases of the airways and precipitate bronchial asthma. Effects of UNIM-352 and optimized preparations of UNIM-352 were assessed on MDA, GSH and SOD levels in blood in OVA sensitized and challenged rats. UNIM-352 and Optimized preparations of UNIM-352 (OP-1, OP-2 OP-3 and OP-4) enhanced the GSH, SOD and reduced the levels of MDA at both the dose levels (200 and 400 mg/kg), when compared with experimental control group.

GSH

Reduced Glutathione (GSH) is a marker of anti-oxidant status and prevents damage to cellular components caused by reactive oxygen species. In bronchial asthma, increased levels of glutathione are typically observed, which appear to relate to the level of pulmonary inflammation which is associated with oxidative stress. Overall analysis of data showed that changes in the level of GSH after various treatments were different across all groups [$F(12, 64) = 35.39$ in blood and $P < 0.01$]. The results showed that sensitization and challenge with OVA resulted in significant decrease in the levels of GSH in blood, as compared to the normal group and the changes were significantly attenuated after treatment with UNIM-352, OP-1, OP-2, OP-3 and OP-4 vs. experimental control group ($p < 0.05$). Higher doses of UNIM-352 (400 mg/kg) and optimized preparations, (OP-1, OP-3 and OP-4 at 400 mg/kg) showed significant increase in GSH levels in comparison to that in experimental control group ($p < 0.01$). The results are summarized in table 1 and figure 1.

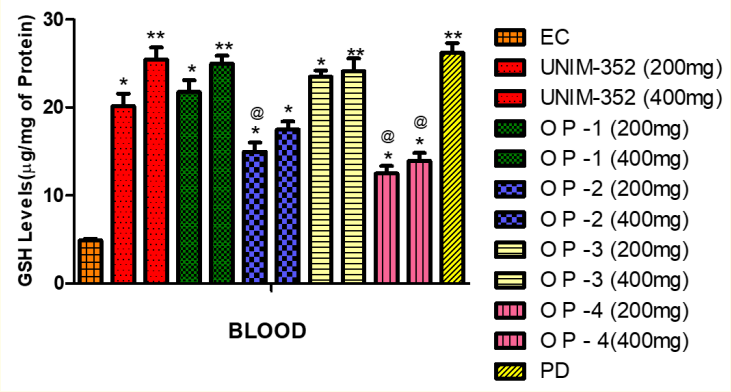

Figure 1: *Effect of UNIM-352 and optimized preparations (OP-1, OP-2, OP-3, OP-4) on GSH levels in blood in OVA sensitized and challenged rats. Data are expressed as Mean ± SEM *p < 0.05, **P < 0.01 vs Experimental control group, @p < 0.05 vs UNIM-352 (200 mg/kg); EC: Experimental control; OP-1 to 4: Optimized preparation 1 to 4; PD: Prednisolone (10 mg/kg); Dose mentioned of different optimized preparation are in (mg/kg).*

MDA

Malondialdehyde (MDA), a marker of lipid peroxidation refers to the oxidative degradation of lipid. It is the process in which free radical steal electron from the lipid in cell membrane resulting in cell damage. Overall analysis of data showed that changes in the level of MDA after various treatments were different across all groups [F 12, 64) = 31.25, P < 0.01]. The results showed that sensitization and challenge with OVA resulted in significant increase in the levels of MDA in blood, as compared to the normal group and the changes were significantly attenuated by the treatment with UNIM-352, OP-1, OP-2, OP-3 and OP-1 vs. experimental control group (p < 0.05 in blood). Higher doses of UNIM- 352 and optimized preparations (OP-1 and OP-3 at 400 mg/kg) showed marked reductions in MDA levels in comparison to that in experimental control group (p < 0.01). Levels of MDA were found to be significantly reduced by the treatment with optimized preparations (OP-1, OP-3 at 400 mg/kg) when compared with UNIM-352 (200 mg/kg) (p < 0.05) and (p < 0.01) respectively. The results are summarized in table 1 and figure 2.

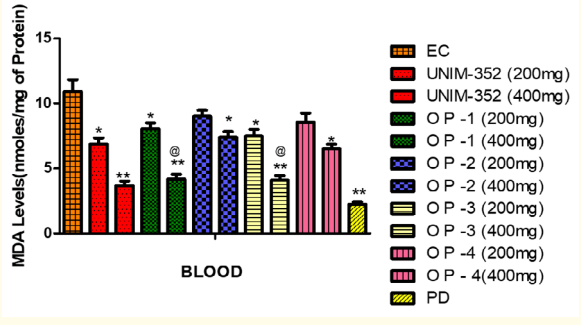

Figure 2: *Effect of UNIM-352 and optimized preparations (OP-1, OP-2, OP-3, OP-4) on MDA levels in blood in OVA sensitized and challenged rats. Data are expressed as Mean ± SEM.*p < 0.05, **P < 0.01 vs Experimental control group, @p < 0.05 vs UNIM-352 (200 mg/kg); EC: Experimental control; OP-1 to 4: Optimized preparation 1 to 4; PD: Prednisolone (10 mg/kg); Dose mentioned of different optimized preparation are in (mg/kg).*

SOD

Effects of UNIM-352 and optimized preparations of UNIM-352 were assessed on SOD levels in blood in OVA sensitized and challenged rats and compared with experimental control. Overall analysis of data showed that changes in the level of SOD after various treatments were different across all groups [F 12, 64) = 7.60, p < 0.01]. The result showed that sensitization and challenge with OVA resulted in significant reduction in SOD levels as compared to normal group and the changes were significantly attenuated by UNIM-352, OP-1 (400 mg/kg), OP-4 (200 mg/kg) and OP-3 200 and 400 mg/kg) vs. experimental control group (p < 0.05). Higher doses of optimized preparations (OP-4 at 400 mg/kg) showed maximal increase in SOD levels vs experimental control group (p < 0.01). The results are summarized in table 1 and figure 3.

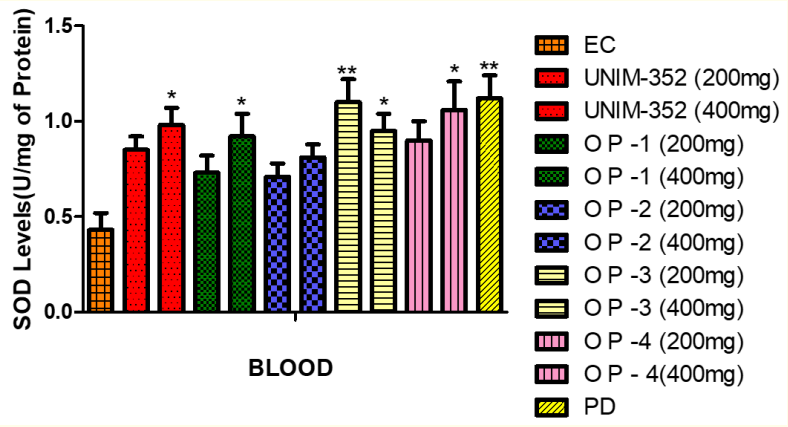

Figure 3: *Effect of UNIM-352, and optimized preparation (OP-1, OP-2, OP-3, OP-4) on SOD levels in blood in OVA sensitized and challenged rats. Data are expressed as Mean ± SEM.*p < 0.05, **P < 0.01 vs experimental control group; EC: Experimental control; OP-1 to 4: Optimized preparation 1 to 4; PD: Prednisolone (10 mg/kg); Dose mentioned of different optimized preparation are in (mg/kg).*

Nitric oxide metabolites (NOx)

Nitric oxide is a gaseous signaling molecule present throughout the body, including in the lungs to fight inflammation and relaxed bronchial muscles. However, the role of nitric oxide is not so simple in asthma and increased in iNOS derived NO mainly to an increased peroxynitrite and imbalance of oxidant and anti-oxidant pathways. Effects of UNIM-352 and optimized preparations of UNIM-352 were assessed on NO levels in blood and compared with vehicle treated OVA sensitized and challenged rats.

Overall analysis of data showed that changes in the level of NO after various treatments were different across all groups [F (12, 64) = 68.37 p < 0.01]. The results showed that sensitization and challenge with OVA resulted in significant reduction by the treatment with UNIM-352 (200 and 400 mg/kg), OP-1 (200 and 400 mg/kg), OP-3 (200 and 400 mg/kg) vs. experimental control group (p < 0.05). Higher doses of optimized formulation (OP-2 and OP-4) at both dose level i.e. 200 and 400 mg showed significant reduction in comparison to that in experimental control group (p < 0.01 in Blood). Levels of NO levels were found significantly decreased by treatment with optimized preparations OP-2 and OP-4 at both dose level i.e. 200 and 400 mg/kg when compared with UNIM-352 (200 mg/kg) (p < 0.05). The results are summarized in table 1 and figure 4.

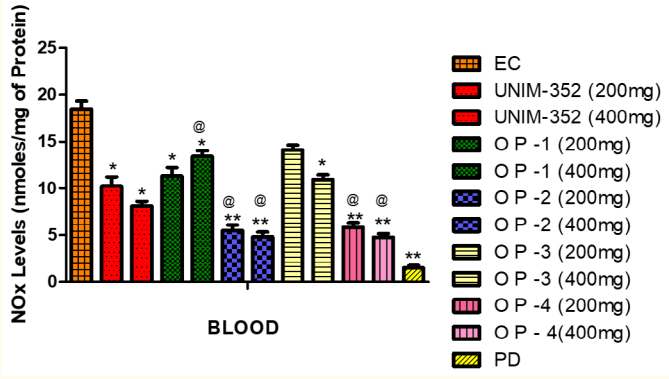

Figure 4: *Effect of Polyherbal formulation UNIM-352, Optimized preparation (OP-1, OP-2, OP-3, OP-4) on NOx levels in blood in OVA sensitized and challenged rats. Data are expressed as Mean ± SEM *p < 0.05, **P < 0.01 vs Experimental control group, @p < 0.05 vs UNIM-352(200); EC: Experimental control; OP-1 to 4: Optimized preparation 1 to 4; PD: Prednisolone (10 mg/kg); Dose mentioned of different optimized preparation are in (mg/kg).*

Effect of UNIM-352 and optimized preparation of UNIM-352 on bronchial hyperresponsiveness to spasmogen in OVA sensitized rats

Bronchial hyperresponsiveness is one of the characteristics features of bronchial asthma. The effect of methacholine inhalation on OVA induced airway hyperresponsiveness was evaluated *in-vivo* 24 hours after last ovalbumin challenge by using whole body plethysmography and represented as enhanced pause (Penh). Penh is an index of bronchial hyperresponsiveness and airflow limitation in experimental animals when using whole body plethysmography. Results showed that values of Penh were significantly increased in experimental control group of rats in response to increasing concentration of methacholine as compared to normal rats. This increased in the value of Penh was reversed after treatment with optimized preparations OP-1 and OP-3 and the results were comparable with prednisolone. The reductions in the value of Penh were more marked with higher dose of OP-1 and OP-3 (400 mg/kg). These results are summarized in table 1 and figure 5.

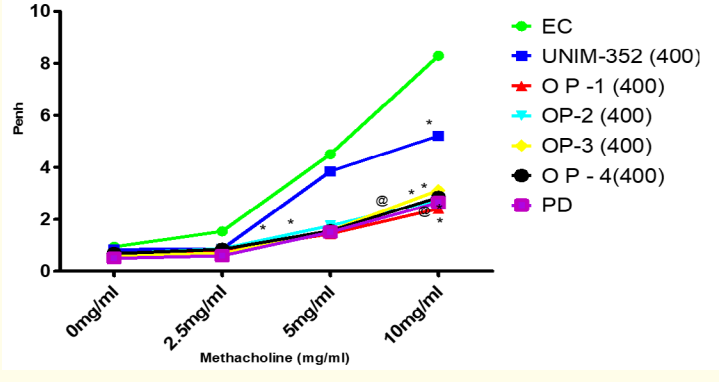

Figure 5: *Effect of polyherbal formulation UNIM-352 and Optimized preparations (OP-1, OP-2, OP-3, OP-4) on bronchial hyperresponsiveness to spasmogen in OVA sensitized rats.Data are expressed as Mean ± SEM *p < 0.05, **P < 0.01 vs Experimental control group, @p < 0.05 vs UNIM-352(200); EC: Experimental control; OP-1 to 4: Optimized preparation 1 to 4; PD: Prednisolone (10 mg/kg); Dose mentioned of different optimized preparation are in (mg/kg).*

Groups	Penh values			
Treatment (mg/kg, p.o.)	At Mch (0 mg/ml)	At Mch (2.5 mg/ml)	At Mch (5.0 mg/ml)	At Mch (10.0 mg/ml)
Normal (Unsensitized)	0.84 ± 0.09	0.95 ± 0.08	1.21 ± 0.09	1.52 ± 0.27
Experimental Control	0.95 ± 0.06	1.54 ± 0.31	4.51 ± 0.78 #	8.29 ± 1.25 #
UNIM 200	1.04 ± 0.08	1.35 ± 0.11	4.23 ± 0.76	6.54 ± 1.51
UNIM 400	0.84 ± 0.06	0.87 ± 0.09 *	3.85 ± 0.56	5.21 ± 1.21
OP-1 (200)	0.61 ± 0.05 * @	0.78 ± 0.09 *	1.58 ± 0.45 *	2.85 ± 0.65 *
OP-1 (400)	0.54 ± 0.04 * @	0.71 ± 0.08 * @	1.45 ± 0.35 * @	2.41 ± 0.45 * @
OP-2 (200)	0.76 ± 0.08	0.91 ± 0.12 *	1.87 ± 0.85	3.85 ± 0.86 *
OP-2 (400)	0.68 ± 0.06	0.88 ± 0.15 *	1.76 ± 0.54	2.75 ± 0.45 *
OP -3 (200)	0.64 ± 0.06 * @	0.69 ± 0.06 * @	1.56 ± 0.56 *	3.12 ± 0.75 *
OP -3 (400)	0.62 ± 0.04 * @	0.65 ± 0.07 * @	1.45 ± 0.65 * @	2.42 ± 0.45 * @
OP -4 (200)	0.82 ± 0.08	0.93 ± 0.09	1.76 ± 0.65	3.54 ± 0.82 *
OP-4 (400)	0.69 ± 0.06	0.85 ± 0.11 *	1.56 ± 0.35 *	2.85 ± 0.65 *
Prednisolone (10)	0.51 ± 0.04 **	0.61 ± 0.06 **	1.51 ± 0.23 *	2.65 ± 0.56 **

Table 1: *Effects of UNIM-352 and optimized preparations of UNIM-352 on enhanced pause (Penh) values to methacholine in OVA sensitized rats.*

*All values are expressed as Mean ± SEM (n = 5/group) $^{\#}p < 0.05$ vs normal group, *P < 0.05, **P < 0.01 vs experimental control group; $^{@}p < 0.05$ vs UNIM-352 (200 mg/kg); UNIM: UNIM-352; OP-1 to 4: Optimized Preparation 1 to 4.*

Discussion

Traditional systems of medicine are widely used globally for the treatment of chronic diseases and both monoherbals and polyherbals are scientifically tested for efficacy and safety-thus opening up new research directions in the integration of traditional and modern medical concepts. The importance of medicinal plant based herbal agents in the treatment of respiratory disorders like asthma has also been emphasized earlier, and the rationale for using polyherbals is being increasingly appreciated-thus necessitating more scientific research in this field [26]. Our current hypothesis was that optimized formulations (with reduced components) could replace the conventional UNIM-352 formulation in the global arena as an effective adjunct to pharmacotherapy of bronchial asthma. In the present study, several novel optimized UNIM-352 formulations with reduced number of components were compared with the conventional UNIM-352 formulation in terms of efficacy and safety. The study also evaluated the potential molecular mechanisms of action of Optimized preparations (OP) of UNIM-352 in experimental models of bronchial asthma.

Complex cellular and molecular mechanisms are involved in asthma pathophysiology and oxidative stress plays pivotal role. Imbalance between pro- and anti-oxidant forces results in raised concentration of ROS and results in oxidative damage to a variety of cellular structures like RNA, DNA and cell membrane [27]. Contact to various stimuli, like allergens, gaseous pollutants, viruses, bacteria, chemicals and drugs results in the permeation and activation of different inflammatory cells in asthmatic airways, such as eosinophils, lymphocytes, mast cells, neutrophils, platelets and macrophages. The acquired immune system response is primarily characterized by the synthesis of IL-5 and the following infiltration activated by allergen-induced responses acting through and activation of eosinophils. On the other hand, stimulation of the innate immune system causes synthesis of IL-8 followed by infiltration and activation of neutrophils. Together, these pathways produce ROS, mainly caused by the respiratory burst of activated eosinophils and neutrophils [28]. Oxidative stress impacts airway by increases in airway hyper reactivity, airway smooth muscle contraction, mucus overproduction, epithelial detachment and increased vascularity. Moreover, reactive

oxygen species induces cytokine and chemokine secretion by activation of oxidative stress-sensitive transcription of NF-κB in airway epithelium. Endogenous antioxidants, catalase, glutathione peroxidase and superoxide dismutase catalytically counteract and deplete reactive oxygen species [29]. Deficiencies of antioxidant vitamins like vitamin-C vitamin-E, and β-carotene are reported in BAL fluid and blood of asthmatic patients. Further, oxidized GSH levels were also shown to be elevated in erythrocytes in asthma. It was suggested that erythrocyte GSH synthesis was increased in response to the presence of increased oxidants and then oxidized to perform its antioxidant role. GSH maintains the redox state of decisive protein sulphydryls that are essential for DNA expression and repair of the nucleus [30]. Reduced GSH in conjunction with glutathione peroxidase and glutathione-S-transferase plays a significant role in defence mechanisms against ROS generated in the biological system. Superoxide dismutase (SOD) belongs to the family of metalloenzymes and dismutases superoxide anion to hydrogen peroxide and oxygen [31]. In the present study, we found that reduced levels of GSH and SOD in OVA challenged rats in both blood and BAL fluid. Treatment with UNIM-352 and optimized preparations of UNIM-352 (OP-3 and OP-4) enhanced the anti-oxidants GSH and SOD levels - suggesting the role of these preparations in reducing the oxidative stress in OVA induced allergic inflammation.

Experimental allergen challenge in bronchial asthma is related with immediate formation of O_2^- present maximum at the site of antigen challenge that persists throughout the late asthmatic phase [32]. Free radicals generated during inflammatory response are short lived in circulation and they are difficult to identify and thus they are assessed indirectly by measuring free radical-mediated lipid peroxidation. Lipid peroxidation is the most important pathophysiological feature resulting in production of secondary metabolite malondialdehyde (MDA), which is marker of oxidative damage in cell membrane. The extent of lipid peroxidation correlates with asthma severity [33]. MDA is a reactive aldehyde produced from polyunsaturated fatty acid and a marker of tissue damage. In our study, OVA inhalation caused increase in the levels of MDA in experimental rats. Treatment with UNIM-352 and optimized formulation of UNIM-352 at different doses showed significant reduction in the levels of MDA in blood - thus suggesting the protective effects of optimized preparations OP-1 and OP-3. Thus, optimized preparations of UNIM-352 may have protective effects against progression of the disease by reducing the oxidative damage caused by reactive oxygen species. Pretreatment with optimized preparations of UNIM-352 at different doses showed significant increase in the antioxidant activity by elevating the antioxidant defence (GSH and SOD) and reducing the lipid peroxidation in OVA induced bronchial asthma model. Nitric oxide (NO) is a multifunctional gasotransmitter which acts as both intercellular and intracellular signaling molecule which is involved in the various pathophysiological and physiological mechanisms in cardiovascular, immune and nervous system. Endogenous NO plays a significant role in the physiological regulation of the airway function which has been implicated in the pathogenesis of the airway diseases like bronchial asthma. NO can increase the allergic inflammatory response by activation of Th2 cells and selective inhibition of Th1 cells. Inducible NOS (iNOS) is reported to be activated by OVA challenge in mouse model and resulted in high levels of NO in BAL fluid. Hence, it is considered to be important marker for the airway inflammation in allergic asthma [34]. Excessive NO combines with superoxides to give peroxynitrite ($OONO^-$), a cytotoxic species that has tissue damaging effects, like lipid oxidation. Consequently, the increased production of reactive oxygen species and reactive nitrogen species by asthmatic subjects may disturb anti-oxidant defense mechanism of body and cause oxidative stress. Thus, in addition to oxidative stress markers, measurement of stable NO metabolite was used as a useful marker to assess treatment outcome with UNIM-352 or the optimized formulations. Our study showed that sensitization and challenge with OVA resulted in significant increase in the levels of NO metabolites (NOx) in blood and treatment with UNIM-352 and the optimized preparations of UNIM-352 (OP-2 and OP-4) significantly reduced NOx levels-suggesting the protective effects of this formulation against such NO formation and its interactions with oxidative markers in this experimental model.

Airway hyper-responsiveness is one of key etiological factors for the characteristic clinical symptoms in patients of bronchial asthma. In airway hyper-responsiveness, there is increased sensitivity of the airways to physical, chemical and pharmacological stimuli like exercise, cold, air, fog, allergen, histamine and methacholine [35]. It is revealed that airways with higher number of eosinophil cells cause various changes in the airway tissue which include, thickening of basement membrane, epithelial damage and release of inflammatory mediators which promotes airway hyper-responsiveness. In asthmatic patients, increased airway smooth muscle mass and sub-epithelial thickening and edema may increase airway resistance [36]. The asthmatic airways contain higher number of mast cells, which are a major source of inflammatory

mediators, like cysteinyl leukotrienes, histamine and prostaglandin D2; and their presence within the airway smooth muscle layer is associated with airway hyper-responsiveness. This is an important characteristic of the asthmatic airways and is associated with loss in epithelial integrity [37]. Whole-body plethysmography (WBP) has been generally used for the measurements of airway hyper-reactivity in conscious and unrestrained animals after airway challenge with allergen and immunization. Bronchial hyper-responsiveness and airflow limitation/restriction is determined by *P-enh* index in experimental animals when using whole body plethysmography. In WBP, changes in airway function are assessed and expressed as an enhanced pause (*P-enh*). The advantage of using *P-enh* system is that animals can be used in a conscious and unrestrained (freely moving) condition. Bronchial hyper-reactivity to inhaled spasmogens such as acetylcholine and methacholine has been assessed by *P-enh* index [38]. Airway responsiveness to non-specific stimuli such as methacholine associated changes has been measured in recent studies. *In-vivo* response to inhaled methacholine is the widely used method of measuring airway hyperresponsiveness and airflow restriction in mice or rats [39]. Results of our experiment showed that values of *P-enh* were significantly increased in experimental control group of rats in response to increasing concentration of methacholine, as compared to normal rats. This increase in the value of *P-enh*, which is a direct correlate of airflow obstruction and restriction, was reversed after treatment with UNIM-352 and optimized preparations OP-1 and OP-3, and these results were comparable with that of prednisolone (comparator drug). The reduction in the value of *P-enh* were more marked with higher dose of OP-1, OP-3 and OP-4 (400 mg/kg), indicating the beneficial effects of optimized preparations of UNIM-352 in bronchial asthma by reversal of bronchial hyper-reactivity and airflow obstruction to increasing concentration of methacholine.

Conclusion

The effect of UNIM-352 and optimized preparations of UNIM-352 were evaluated on oxidative stress markers and bronchial hyperreactivity in the OVA immunized model of allergic asthma in rats. UNIM-352 and its optimized (OP) preparations showed differential degree of attenuations in MDA (OP-1 and OP-3) and NOx (OP-2 and OP-4) as compared to experimental control group of rats. On the other hand, greater augmentations in the levels of GSH and SOD in blood were seen with optimized preparations OP-3 and OP-4 when compared with conventional UNIM-352. This showed the greater effects of optimized preparation of UNIM-352 against oxidative and nitrosative stress markers in OVA-induced model of airway inflammation. Bronchial hyperreactivity and airflow restriction to methacholine challenge was also differentially attenuated after OP UNIM-352 formulations by differing degrees. Thus the optimized formulations were equally efficacious as compared to the conventional UNIM-352 formulation, and could be proposed as alternatives for the treatment of bronchial asthma - thereby highlighting the translational significance of this research.

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