

## Effect of Breath<sup>o</sup>In On Oxidative Pulmonary Damage and Fibrosis in A Rodent Model

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### Abstract:

**Aim:** The present study evaluated the protective effects of Breath<sup>o</sup>In, on oxidative damage and fibrosis in the lungs of rats.

**Method:** Pulmonary damage and fibrosis was induced in female Sprague Dawley rats using CCl<sub>4</sub> (3 ml/kg b.w. i.p., 20% v/v CCl<sub>4</sub>/olive oil) for 28 days. The role of Breath<sup>o</sup>In in reversing the effects of CCl<sub>4</sub> was studied by measuring the levels of superoxide dismutase, catalase, glutathione and collagen in lung tissues.

**Results:** The results showed that CCl<sub>4</sub> exerted its toxic effects on lung tissue by decreasing the levels of SOD, CAT and GSH. In contrast, co-administration of Breath<sup>o</sup>In reversed the CCl<sub>4</sub> induced decreases in SOD, CAT and GSH levels. While CCl<sub>4</sub> led to a significant increase in collagen deposition, treatment with Breath<sup>o</sup>In led to a significant decrease in collagen deposition. These results were in agreement with results obtained when rats were co-treated with prednisolone. Thus, the results of this study indicate that Breath<sup>o</sup>In has the ability to reduce oxidative damage and fibrosis in lung tissues.

**Conclusion:** Our findings suggest that Breath<sup>o</sup>In demonstrates protective properties and is effective in reducing pulmonary damage that was induced by oxidative stress produced by CCl<sub>4</sub>.

**Keywords:** Breath<sup>o</sup>In, collagen, pulmonary damage, fibrosis and CCl<sub>4</sub>.

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### I. INTRODUCTION

Pulmonary fibrosis ("scarring of the lungs") is a respiratory disease in which scars are formed in the lung tissues, leading to serious breathing problems. Scar formation, with the accumulation of excess fibrous connective tissue (fibrosis), leads to thickening of walls, resulting in reduced oxygen supply in the blood. As a consequence, patients suffer from perpetual shortness of breath.

Pulmonary fibrosis presents itself clinically by a history of progressive shortness of breath (dyspnea) during exertion. Sometimes fine inspiratory crackles can be heard at the base of the lungs on auscultation. Pulmonary fibrosis may also be a secondary effect due to other diseases. Most of these are classified as interstitial lung diseases. Examples include autoimmune disorders, viral infections and bacterial infection like tuberculosis which may cause fibrotic changes in upper or lower lobes of the lungs and other microscopic injuries to the lung.

In some patients, the specific cause of the disease can be diagnosed. However, in cases where pulmonary fibrosis can appear without any known cause, the condition is called idiopathic pulmonary fibrosis. In these cases, there is no known cure for the scars and damage that occurs to the lungs due to pulmonary fibrosis.

The mechanism underlying the pathogenesis of pulmonary fibrosis involves multiple pathways, such as inflammation, oxidative stress, and developmental processes. These can result in alveolar epithelial cell injury and fibroblast proliferation, consequently leading to abnormal deposition of extracellular collagen [1]. These inflammatory cells can synthesize and secrete various cytokines, chemokines, reactive oxygen species, and proteases, which can lead to aberrant fibro-proliferation and collagen production [2]. Disturbances in cytokine expression are involved with the increase in free radical production. The enhanced production of free radicals and oxidative stress can also be induced by a variety of factors such as radiation or exposure to heavy metals and exposure to xenobiotics [3].

Carbon tetrachloride (CCl<sub>4</sub>) is one such xenobiotic that has been demonstrated to cause injury to the lungs [4]. It has been proven that CCl<sub>4</sub> promotes injury via oxidative stress by increasing the lipid peroxidation and lowering the endogenous antioxidant. Common diseases in the lung such as asthma, chronic obstructive pulmonary disease and cystic fibrosis have been demonstrated to share similar pathogenesis, i.e., increased production of reactive oxygen species, which correlates with disease severity [5].

## II. MATERIALS AND METHODS

Breath'In is a polyherbal formulation developed by Suguna Lifeherbs, Herbal division, Suguna Foods Pvt. Ltd. 169/1(P), Kottamangalam village, Tirupur Main Road, Madathukulam Taluk, Tirupur District, TN- 642202

### Chemicals and drugs:

Standard drugs such as prednisolone were obtained from the medical store. Standard solvents and chemicals (CCl<sub>4</sub>, olive oil, phosphate buffer, sodium carbonate, Triton X, Nitroblue tetrazolium (NBT), hydroxylamine hydrochloride, EDTA, hydrochloric acid, copper sulphate, hydrogen peroxide, p-dimethyl amino benzaldehyde, DTNB (Ellman's reagent), TCA, sodium citrate, potassium dichromate, acetic acid, etc.) were obtained from the college stores.

**Experimental animals:** Swiss Albino mice (n=6, weighing 18-25 g) and female Sprague Dawley rats (n=24, weighing 170-200 g) were used in these studies. All the animals were procured from Adita Biosys Pvt. Ltd., Tumakuru, CPCSEA Registration No: 1868/PO/Bt/S/16/CPCSEA (with health certificate of the animals), and were maintained under controlled condition of temperature (23 ± 2°C), humidity (50±5%) and 12-hour light and dark cycles. The animals were randomized into experimental and control groups and housed in sanitized polypropylene cage containing sterile paddy husk as bedding. They had free access to standard food pellets and water. Assignment of animals to experimental and control groups was made in such a way that each group had mean and total body weights like the other groups. The acute oral toxicity study was carried out according to the guidelines established by OECD. An ethical clearance was obtained from the Institutional Animal Ethics Committee (PESCP/IAEC/33/2016) and the study was conducted according to the guidelines of CPCSEA, New Delhi.

### Acute oral toxicity study:

Swiss Albino mice were used for the acute toxicity study. The purpose of this study was to determine the LD50 of Breath'In. Based on the results of this study, the median dose of Breath'In was selected and used for the remainder of the experiments.

Prior to dosing, animals were fasted overnight, weighed and the dose calculated according to the body weight. Single animals were dosed in sequence usually at 48 h intervals. Using the default progression factor, doses were selected from the sequence 175, 550, 2000 and 5000 mg. As no estimate of Breath'In lethality was obtained, dosing was initiated at a dose of 175 mg/kg till 5000 mg/kg as recommended in the OECD Guidelines 425 [6]. The LD50 was calculated by the changes in the observations for the main test at 5000 mg/kg body weight.

**Table 1:** Observation of acute oral toxicity at 5000 mg/kg dose of Breath'In (single dose)

Observation At	30 min	4 h	24 h	48 h
Skin and fur	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Convulsion	Nil	Nil	Nil	Nil
Mortality	Nil	Nil	Nil	Nil

The results showed that no mortality occurred at a dose of 5000 mg/kg b.w. This observation was extended for an additional 14 days. While 1/10 of this does (500 mg/kg) was warranted, the study selected a dose of 300 mg/kg b.w. as the median dose to avoid mid study mortality and to have safer indications.

### Experimental design for the evaluation of Breath'In on lung parameters in rats:

The rats were divided into four groups containing six rats in each group. Prior to the start of the experiment, all the rats were housed individually in stainless steel mesh cages with individual food cups for weighed diets and they were housed in light-controlled room (12 h light / dark cycles) with free access to drinking water. All the animals were maintained on a control diet for 7 days as an acclimatization period and then regrouped based on their feeding pattern.

Group I animals were fed a normal pellet diet and received water and olive oil (3 mg/kg i.p.) for 28 days. Group II received normal pellet diet along with CCl<sub>4</sub> (3 mg/kg b.w. i.p.), and 20% v/v CCl<sub>4</sub>/olive oil.

Group III were administered Breath'In (300mg/kg p.o.) for 28 days along with CCl<sub>4</sub> (3 mg/kg b.w. i.p.), and 20% v/v CCl<sub>4</sub>/olive oil, given for 28 days twice a week (3, 6, 10, 13, 17, 20, 24 and 28<sup>th</sup> day). Group IV received prednisolone (20 mg/kg b.w. orally) for 28 days along with CCl<sub>4</sub> (3mg/kg b.w. i.p.), and 20% v/v CCl<sub>4</sub>/olive oil, for 28 days twice a week (3, 6, 10, 13, 17, 20, 24 and 28<sup>th</sup> day).

### III. ESTIMATION OF TISSUE PARAMETERS

**Tissue processing:** Tissue processing was done 24h after the last dose of treatment. The animals were sacrificed as per the CPCSEA guidelines and the lungs were isolated and weighed. The homogenates were prepared on ice in the ratio of 4g of tissue with 16 ml of phosphate buffer (pH 7.5), containing 1 mM/l disodium EDTA and 10 ml of 500mM/l BHT in acetonitrile to prevent the formation of new peroxides during the assay. The homogenates were centrifuged at 4° C at 2000 g for 20 min [6]. The resultant homogenates were used for the estimation of superoxide dismutase, catalase, glutathione and collagen content.

**Estimation of Superoxide Dismutase (SOD):** SOD was estimated according to method of Kono [7]. The reaction mixture containing 1.3 ml sodium carbonate buffer, 500 µl Nitroblue Tetrazolium chloride (NBT) and 100 µl Triton X-100 was taken in a test cuvette. The reaction was initiated by the addition of 100 µl hydroxylamine hydrochloride. After 2 min, 70 µl of the enzyme extract was added. The percentage inhibition in the rate of NBT reduction was recorded as an increase in absorbance at 540 nm [8].

**Estimation of Catalase (CAT):** The estimation of CAT was based on the method of Sinha [9]. The assay mixture contained 4 ml of H<sub>2</sub>O<sub>2</sub> (800µM) and 5ml of 0.01 M phosphate buffer in a test tube. 1 ml of a properly diluted portion of the enzymatic reaction mixture was mixed rapidly in a swirling motion gently, allowing it to mix thoroughly. Then 1 ml of this reaction mixture was withdrawn and blown into 2 ml of the dichromate/acetic acid reagent. The samples were heated in boiling water for 10 min. The total volume of the reaction mixture was made to 3 ml and the optical density/ absorbance was measured at 570nm against color/blank [9].

**Estimation of Glutathione (GSH):** The assay is based upon the principle of Ellman's reagent, 5-5'-Dithio-bis 2- nitrobenzoic acid (DTNB or Ellman's reagent). GSH was measured by its reaction with Ellman's reagent to yield a yellow chromophore which was measured spectrophotometrically. The lung homogenate was mixed with an equal volume of 10% TCA (trichloroacetic acid) and was centrifuged at 4000 g rpm for 5 min at 4° C. The supernatant thus obtained was used for the GSH estimation. To 0.1 ml of the processed tissue sample, 2 ml of phosphate buffer, 0.5 ml of DTNB and 0.4 ml of distilled water was added to the mixture and was shaken vigorously on vortex. The absorbance was measured at 412 nm within 15 min [10, 11].

**Hydroxyproline assay:** The Hydroxyproline assay was done to estimate the amount of collagen content in the lungs. The lungs were cleaned and stored in 10% formalin. They were hydrolysed with 0.3ml of 6M HCl for 24 h at 110°C followed by oxidation with 0.1N Chloramine-T. The homogenate thus obtained was used for the estimation of hydroxylamine. In different test tubes, 1 ml of standard and sample solution was added. 1 ml of 0.05M copper sulphate solution and 1 ml of 2.5N sodium hydroxide solutions were added. Tubes were placed on water bath for 5 min at 40°C and cooled for 10 min. 1 ml of 6% H<sub>2</sub>O<sub>2</sub> was added and again boiled in water bath for 10 min. Tubes were cooled and 4 ml of 3N sulphuric acid and 2 ml of 5% p-dimethyl amino benzaldehyde solution was added and boiled at 70°C for 16 min in water bath. The samples were cooled, and absorbance of both standard and experimental samples was measured at 555 nm [12].

### STATISTICAL METHODS:

All experiments were performed in triplicates. All data are expressed as the standard error of the mean (S.E. ±mean). Comparisons among the control and treatment groups were made using analysis of variance (ANOVA) followed by a Bonferroni method of Statistics using the Graph pad prism statistical program. The results were considered statistically significant if 'p' value was =05 or less.

### IV. RESULTS

The present study evaluated the protective effects of Breath'In, on oxidative damage and fibrosis in the lungs of rats. Pulmonary damage and fibrosis was induced in Sprague Dawley rats by the administration of CCl<sub>4</sub> (3 ml/kg b.w. i.p., 20% v/v CCl<sub>4</sub>/olive oil) for 28days. Lung tissue was evaluated for parameters such as SOD, CAT, GSH, and collagen using previously published assays.

**Table 2:** Effects of Breath'In on super dismutase (SOD) levels

Groups (n=6)	SOD (U/g tissue)
I. Normal	44.24±3.234

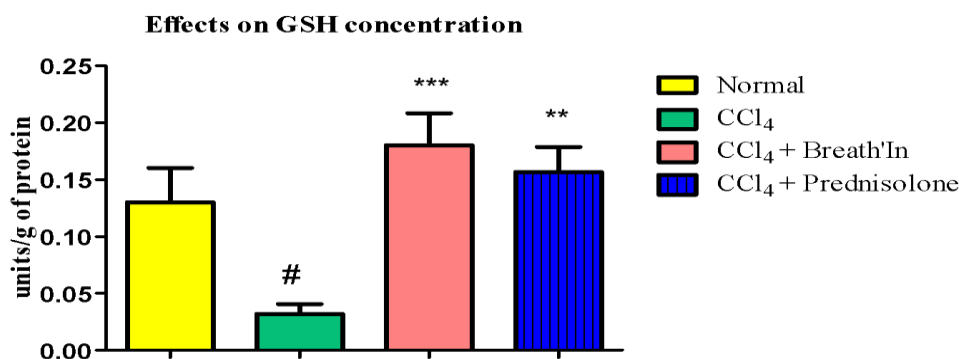
II. CCl <sub>4</sub>	38.08±1.086###
III. CCl <sub>4</sub> + Breath'In	41.79±2.073*
IV. CCl <sub>4</sub> + Prednisolone	42.64±1.631**

All data are expressed as the standard error of the mean (S.E. ±mean). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 is considered significant when compared to CCl<sub>4</sub> group; #p<0.05, ##p<0.01, ###p<0.001 is considered significant when compared to control group 1.

**Table 3:** Effects of Breath'In on catalase (CAT) levels

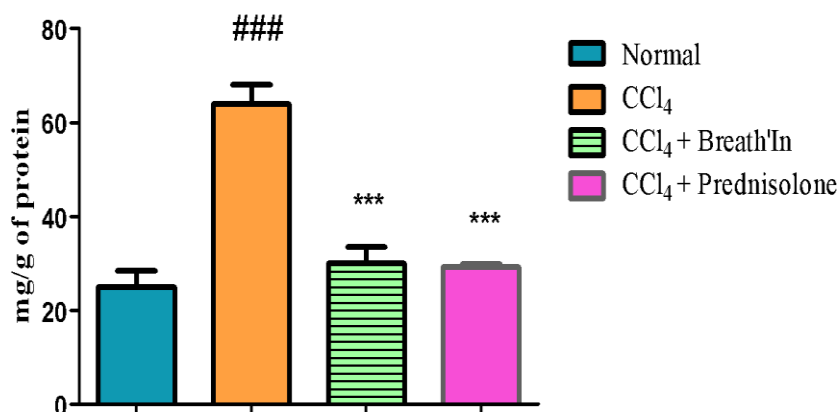
Groups (n=6)	CAT (U/μM H <sub>2</sub> O <sub>2</sub> per min)
I. Normal	164.3±2.501
II. CCl <sub>4</sub>	155.6±3.517###
III. CCl <sub>4</sub> + Breath'In	169.3 ± 1.465***
IV. CCl <sub>4</sub> + Prednisolone	167.7 ±1.348***

All data are expressed as the standard error of the mean (S.E. ±mean). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 is considered significant when compared to CCl<sub>4</sub> group; #p<0.05, ##p<0.01, ###p<0.001 is considered significant when compared to control group 1.



**Fig 1:** Effects of Breath'In on glutathione (GSH) levels

All data are expressed as the standard error of the mean (S.E. ±mean). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 is considered significant when compared to CCl<sub>4</sub> group; #p<0.05, ##p<0.01, ###p<0.001 is considered significant when compared to control group 1.



**Fig 2:** Effects of Breath'In on collagen levels

All data are expressed as the standard error of the mean (S.E. ±mean). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 is considered significant when compared to CCl<sub>4</sub> group; #p<0.05, ##p<0.01, ###p<0.001 is considered significant when compared to control group 1.

## V. DISCUSSION

The results obtained from this study suggest that Breath'In, a polyherbal formulation, appears to reduce the pulmonary and fibrotic damage caused by CCl<sub>4</sub> induced oxidative stress in rats [13]. This observation is based on the effects of Breath'In on several measured parameters.

SOD is an enzyme that works as an antioxidant and plays a key role in protecting the lung tissue from oxidative damage. Hence, depletion in SOD levels clearly marks the damage caused to the lung tissue. In this study, animals treated with CCl<sub>4</sub> showed a marked decrease in the level of SOD as compared to the control animals. Given that CCl<sub>4</sub> is a toxic agent which causes oxidative damage to the lungs, it decreases the levels of the marker enzyme, SOD. Co-administration of Breath'In reversed the decreases in SOD levels caused by CCl<sub>4</sub>. It was significant to note that the levels of SOD following treatment with Breath'In were similar to those observed when animals were administered prednisolone, a standard drug known to protect against pulmonary damage and fibrosis.

CAT is an antioxidant enzyme which plays a key role in detoxification of the lungs from damage caused due to oxidation and formation of ROS or reactive oxygen species. These ROS are produced from CCl<sub>4</sub> (trichloromethyl radical ( $\cdot$ CCl<sub>3</sub>) and peroxytrichloromethyl radical ( $\cdot$ OOCCl<sub>3</sub>), which cause oxidative damage in the lungs [14]. In this study, a significant reduction in CAT activity was seen in animals treated with CCl<sub>4</sub> compared to control animals. Interestingly, animals treated with Breath'In along with CCl<sub>4</sub> showed a protective action by increasing the levels of CAT activity, similar to animals treated with the standard drug prednisolone in the presence of CCl<sub>4</sub>.

Treatment with CCl<sub>4</sub> leads to the generation of free radicals which increase lipid peroxidation and elevate thiobarbituric acid, while depleting the tissue content of GSH [12]. The results showed that GSH content in the tissues were drastically depleted by the induction of CCl<sub>4</sub> compared to the control group of the study. In contrast, co-treatment with Breath'In in the CCl<sub>4</sub> treated animals showed a significant increase in GSH levels in lung tissues. Similarly, a significant increase in the GSH content was observed in animals treated with the standard drug prednisolone along with CCl<sub>4</sub>.

The hydroxyproline assay was done to evaluate the content of collagen in rat lungs following treatment with CCl<sub>4</sub> compared to normal controls and rats treated with Breath'In. A significant increase in collagen deposition was observed following 4 weeks of CCl<sub>4</sub> treatment compared to control animals. When animals treated with CCl<sub>4</sub> were also treated with Breath'In, it was observed that Breath'In provided a protective effect with a significant decrease in collagen deposition. Thus, the results suggest that Breath'In appeared to counter the fibrotic effects of CCl<sub>4</sub> in these animals. A similar result was observed in animals treated with prednisolone and CCl<sub>4</sub>.

## VI. CONCLUSION

The results obtained from this study suggest that Breath'In, a polyherbal formulation exhibited a protective effect on several lung parameters in rats. Breath'In appears to exert an antioxidant and protective effect on lungs as it increased the antioxidant marker enzyme SOD, antioxidant CAT and GSH content in lung tissue. It also decreased the collagen content, which is a salient parameter in fibrotic lung diseases. This study, using a rodent model, suggests that Breath'In may be a useful herbal formulation to alleviate respiratory problems related to pulmonary fibrosis, chronic obstructive pulmonary diseases, asthma and cystic fibrosis. Further studies aimed at the mechanism by which Breath'In exerts its protective effects are necessary before definite conclusions can be drawn. However, the data provide useful insights into the possibility of using Breath'In for the treatment of several respiratory related disorders.

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