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ORIGINAL ARTICLE

Celastrus paniculatus Seed Oil Ameliorates Oxidative Stress in Lipopolysaccharide-Induced Respiratory Inflammation in Mice

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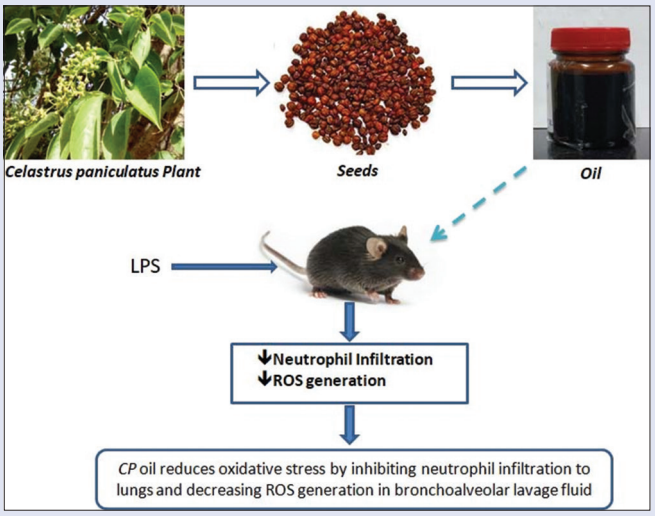
ABSTRACT

Background: *Celastrus paniculatus* have been reported to possess various medicinal properties. However, very little is known about its effect on respiratory inflammation. **Objectives:** We evaluated *Celastrus paniculatus* seed oil (CP oil) on lipopolysaccharide (LPS)-induced mice model of respiratory inflammation. **Materials and Methods:** Both short-term and long-term studies were carried out. In short-term study, C57BL/6 mice were exposed to LPS (10 mg/kg) by intranasal route for one week. CP oil (1 g/kg) was given orally on the 1st, 3rd and 5th day. All the mice were sacrificed on the 7th day. In long-term study, C57BL/6 mice were exposed to LPS (10 mg/kg) by the intranasal route for one month. CP oil (1 g/kg) or theophylline (1 mg/kg) was administered twice a week for one month. At the end of the treatment, Bronchoalveolar lavage (BAL) fluid was collected and labelled with CD3e FITC, Ly-6G (Gr-1) PE-Cy-7, Allergin-1 eFlour® 660, CD14 APC and 2′,7′-dichlorofluorescein diacetate (20 μM) and analyzed by flow cytometer (BD FACSCanto™ II) for cellular infiltration and reactive oxygen species (ROS) generation. There was a significant reduction in neutrophil infiltration following CP oil treatment for one month (68±6% vs 40±7%, $P < 0.05$). Furthermore, there was a significant decrease in ROS generation following CP oil treatment for one week (5873 ± 1133 vs 2581 ± 1359; $P < 0.05$) and one month (20618 ± 1854 vs 5850 ± 1006; $P < 0.05$). Further, theophylline treatment for one month reduced the ROS generation significantly (20618 ± 1854 vs 5286 ± 2413; $P < 0.05$). **Conclusion:** *Celastrus paniculatus* seed oil seems to reduce oxidative stress by inhibiting the generation of reactive oxygen species in LPS-induced lung inflammation.

Keywords: *Celastrus paniculatus*, COPD, oxidative stress, reactive oxygen species, respiratory inflammation

SUMMARY

- *Celastrus paniculatus,* which is known as jyotishmati in ayurveda is a wildly grown woody climber. Oil from seeds of *C. paniculatus* is well documented to offer protective effects against ulcers, skin diseases, wounds, inflammation, cardiac problems, gastrointestinal diseases and neurodegenerative diseases. We studied for the beneficial effect of *C. paniculatus* seed oil on lipopolysaccharide-induced mice model of respiratory inflammation which simulate COPD and asthmatic conditions. *C. paniculatus* oil was found to decrease the generation of reactive oxygen species in bronchoalveolar lavage fluid in lipopolysaccharide-induced mice. This suggests that *C. paniculatus* seed oil may act as a better therapeutic for treatment of asthma, COPD. and respiratory inflammation.



Abbreviations used: APC: Allophycocyanin; ANOVA: Analysis of variance; BAL: Bronchoalveolar lavage; cAMP: Cyclic Adenosine monophosphate; CD14: Cluster of differentiation 14; CD3e: Cluster of differentiation 3epsilon; CP oil: *Celastrus paniculatus* seed oil; COPD: Chronic obstructive pulmonary disease; DCF-DA: 2′,7′–dichlorofluorescin diacetate; DMEM: Dulbecco's Modified Eagle Medium; FITC: Fluorescein isothiocyanate; GC-MS: Gas chromatography–mass spectrometry; H_2O_2: Hydrogen peroxide; LPS: Lipopolysaccharide; MFI: Mena fluorescence index; NADPH: nicotinamide adenine dinucleotide phosphate; O_2–: Superoxide ion; ROS: Reactive oxygen species; PE: Phycoerythrin; RBCs: Red blood cells; SEM: Standard error of mean.

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INTRODUCTION

Respiratory diseases like asthma and chronic obstructive pulmonary disease (COPD) are the leading causes of morbidity and mortality across the globe. Shortness of breath, chest tightness, airway obstruction, and chronic inflammatory response in the airways are the hallmarks of COPD.[1] Around 4.36% of the Indian rural sub-populations above 35 years of age have been reported to have developed COPD, according to a study.[2] Also, another study shows that the overall prevalence of COPD in India among adults above 35 years of age is 3.49%.[3] All these studies suggest a significant COPD disease burden among the Indian population.

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Recently, many phytochemicals exhibiting anti-inflammatory activities showed promising protective activity against elastase and lipopolysaccharide (LPS)-induced mice model of lung inflammation.[4] This shows that the use of phytochemicals to inhibit the inflammatory responses in respiratory diseases like COPD and asthma seems to be an attractive therapeutic option.[5]

Celastrus paniculatus belongs to the family *Celastraceae* and is known as jyotishmati in ayurveda. It is a critically endangered woody climber of about 10–18 m in height.[6] *C. paniculatus* commonly grows all over the hilly parts of India where there is tropical dry deciduous forest.[7] However, it is also found to grow in various climates and environments in various parts of the world like the Pacific islands, Nepal, Sri Lanka, Thailand, Vietnam, Indonesia, Malaysia, Myanmar, Australia, and China.[8,9] Oil extracted from the seeds of *C. paniculatus* is dark, reddish-brown, is quite useful, and has medicinal properties.

Also, *C. paniculatus* seed oil is reported to be used as a diuretic, emmenagogue, diaphoretic, febrifuge, digestive, laxative, appetizer, expectorant, and powerful brain tonic in ayurvedic medicine.[10] Furthermore, it has been reported to be used against ulcers, scabies, pruritis, skin diseases, wounds, leukoderma, cephalalgia, arthralgia, asthma, cardiac debility, inflammation, amenorrhea, dysmenorrhea, and epilepsy.[11-14]

Moreover, *C. paniculatus* seed oil is reported to be a potent scavenger of reactive oxygen species. Reactive oxygen species (ROS) from both endogenous and exogenous sources are involved in ageing, age-associated neurodegenerative diseases and cognitive deficits. *C. paniculatus* seed oil has exhibited protective effects on cognition and neurodegenerative diseases in various *in vitro* studies and animal models.[15,16] However, the effect of *C. paniculatus* seed oil on inflammatory respiratory diseases has not been studied yet. Therefore, we have evaluated the effect of *C. paniculatus* seed oil (CP oil) on oxidative stress on LPS-induced mice model of lung inflammation in this study.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide from *Escherichia coli* 055:B5, Tris Buffer, Hydrogen Peroxide, 2',7'-Dichlorofluoroscein Diacetate (DCF-DA), Theophylline and Dulbecco's Modified Eagle Medium (DMEM) were procured from Sigma (Sigma-Aldrich Inc., St. Louis, USA). Oil from *Celastrus paniculatus* seed was procured locally. A sample of *C. paniculatus* seed oil has been kept in the laboratory for future references. Also, a reference sample of pure *C. paniculatus* seed oil was procured from Deve Herbes, India for comparative GC-MS analysis. Anti-mouse CD3e FITC, Anti-mouse allergin-1 eFlour® 660, Anti-mouse CD14 APC and Anti-mouse Ly-6G (Gr-1) PE-Cy7 were procured for labelling T cell, mast cells, macrophages and neutrophils respectively from eBioscience (eBioscience, Inc, USA). All the other chemicals used were of either analytical or molecular grade.

Gas chromatography-mass spectrometry (GC–MS) analysis

Comparative GC–MS analysis of *C. paniculatus* seed oil obtained locally and *C. paniculatus* seed oil procured from Deve Herbes, india was performed on Agilent 8890 GC system, 7000D TQ MS system. Agilent HP-5MS UI column (30 m × 250 µm × 0.25 µm) was used for the analysis. Components were analyzed using He as carrier gas at a constant flow of 1 ml/min. The operating conditions of the column were as follows: Oven temperature was set at 60°C initially, with a hold time of 2 min. A gradual increase from 60°C to 300°C at 10°C/min with a hold time of 2 min, and a run time of 29 min was then carried out. The injector temperature was maintained at 250°C, 1 µl sample was injected at a constant pressure of 8.2317 psi, the total flow was 27 ml/min and column flow were set as 1 ml/min. Average velocity 44.635 cm/s, (septum) purge flow 3.0 ml/min, split ratio 20:1; ion source temperature (230°C to maximum 250°C). Fragments were from 30 to 700 Da. Spectrum obtained were compared with the GC-MS NIST library database.

Animals

Experiments were performed on male C57BL/6 mice (average weight, 23 g). All the animals were procured and kept in polypropylene cages and maintained at 24°C ± 0.5°C, 12 hr day/night cycle with relative humidity (55+10%), in the Central Animal Facility of the Indian Institute of Science, Bangalore and were provided with chow pellets and water *ad libitum*. All the experiments were performed according to the guidelines of the Committee for Control and Supervision of Experiments on Animals and prior approval was obtained from the Institute Animal Ethics Committee of the Institute (CAF/Ethics/194/2010).

Experimental design and animal treatment

In the first experiment, the mice were treated for one week (short-term exposure and treatment). C57BL/6 mice were divided into groups, each group having 4 mice each: the control group (group I), LPS administered group (group II), and *C. paniculatus* oil group (group III). Group III received (1 gm/kg) *C. paniculatus* oil orally on 1st, 3rd and 5th day. All the groups except group I received intranasal LPS (10 mg/kg) instillation on the 2nd, 4th, and 6th day. All the mice were sacrificed on the 7th day.

In second experiment, the mice were treated for one month (long-term exposure and treatment). C57BL/6 mice were divided into four groups, each group having 4–5 mice each: the control group (group I), LPS administered group (group II), and *C. paniculatus* oil group (group III), and theophylline-treated group (group IV). Group III received CP oil (1 gm/kg) orally, twice a week for one month, and group IV received theophylline (1 mg/kg) intraperitoneally per mice, twice a week for one month. All the groups except group I received intranasal LPS (10 mg/kg) installation twice a week for one month. Intranasal LPS instillation was carried out on the next day of *C. paniculatus* oil or theophylline administration. All the mice were sacrificed at the end of one month for further studies.

Bronchoalveolar Lavage fluid collection

The bronchoalveolar lavage (BAL) fluid was collected as per the methods described by Edwan JH, *et al.*, and Heeckeren A, *et al.* with slight modifications.[17,18] Briefly, phosphate buffer saline (2 ml) was infused through the trachea into the lungs by a cannulated syringe, and bronchoalveolar lavage fluid was aspirated out slowly and gently. This process was repeated five times and ≥8 ml of BAL fluid lavage was collected from each mouse. Thereafter, the collected BAL fluid was centrifuged at 350 g for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in the phosphate buffer saline (pH 7.4) for further studies. Any RBCs in the pellet was removed by treatment with ammonium chloride RBC lysis buffer (pH 7.4). The cells were taken for further studies.

Measurement of reactive oxygen species

Assay of reactive oxygen species (ROS) generation was carried out as per the methods described by Porto BN, *et al.* with slight modifications.[19] Briefly, three sets of microtubes for each group (control group, LPS treated group, CP oil, and theophylline) were taken and 200 µl (10^6 cells/

microtube) of BAL fluid cells resuspended in PBS were incubated with 2',7'-dichlorofluorescein diacetate (DCF-DA) (20 μM) that was converted into highly fluorescent 2',7'-dichlorofluorescein presence of cellular peroxides including H_2O_2. The first group of tubes were taken as control; to the second group of tubes only DCF-DA (20 μM) was added, whereas to the third group of tubes DCF-DA (20 μM) and H_2O_2 (20 μM) was added. The tubes were incubated at room temperature for 30 min in the dark following the addition of DCF-DA. Thereafter, the tubes were incubated at room temperature for 15 min in the dark following the addition of H_2O_2. Thereafter, these were transferred to flow cytometry tubes and acquired in a flow cytometer (BD FACSCanto™ II) and analyzed using FACSDiva Software (version 6.1.3). Mean fluorescence intensity was taken as a parameter for ROS generation.

Measurement of cellular infiltration and antibody labeling

Measurement of cellular infiltration and antibody labelling was done per the methods described by Surin WR *et al.*[20] Briefly, 1 × 10^7 cells were taken and labelled with various antibodies (Anti-mouse CD3e FITC, Anti-mouse Allergin-1 eFlour® 660 Anti-mouse Ly-6G (Gr-1) PE-Cy-7 and Anti-mouse CD14 APC for labelling T cell, mast cells, neutrophils and macrophages) as per the manufacturer's instruction (eBioscience, Inc. San Diego, USA). The cells were incubated for about one-and-a-half hours at 2°C–8°C on ice in the dark. The cells were then transferred to FACS tube and acquired in a flow cytometer (BD FACSCanto™ II) and analyzed using FACSDiva software (version 6.1.3).

Statistical analysis

All the assays were performed in duplicates of ≥3 independent experiments. The percentage of infiltration from each group as well as the mean fluorescence index (MFI) for ROS generation was collated to derive the mean and standard error of the mean (SEM). Simultaneously, analysis of data was performed by one-way ANOVA followed by *post hoc* Dunnett's test concerning control group by GraphPad Prism statistical software (GraphPad Software, San Diego, USA). All the data are reported as mean ± SEM in all the groups. $P < 0.5$ was considered to be statistically significant.

RESULTS

Analysis of Bronchoalveolar Lavage Fluid

Effect on the infiltration of T cells

It is well established that there was an increase in the level of T cells during chronic lung inflammation.[21] There was a significant increase in the level of T cells in the BAL fluid following one-week LPS exposure (24±4% vs 57±12%) [Figure 1]. However, CP oil administration did not reduce the level of T-cell population in BAL fluid during short-term treatment (57±12% vs 36±9%) [Figure 1]. Also, there was a significant increase in the level of T cells following long-term exposure to LPS (34±5% vs 63±10%) [Figure 2]. Interestingly, CP oil did not reduce the level of T-cell population in BAL fluid during long-term treatment [Figure 2]. However, there was a significant reduction in the infiltration of T cells in the theophylline administered group (63±10% vs 25±12%; $P < 0.05$) [Figure 2].

Effect on the infiltration of mast cells

Mast cells play a primary role and initiate an appropriate program of inflammation and repair in response to tissue damage caused by a variety of diverse stimuli.[22] Therefore, we analyzed the level of mast cells in BAL fluid via flow cytometry. We observed no significant increase in the level of mast cells in the LPS-treated group from that of control (25±6% vs 35±7%) [Figure 1]. However, there was a marginal increase in the level of mast cells in the BAL fluid of CP oil–treated animals (25±6% vs 46±9%) [Figure 1]. Also, when we evaluated the percentage of mast cells across all the experimental groups with long-term treatment, we observed no significant increase in the level of mast cells either in LPS- (27±9% vs 36±12%) or in CP oil–treated (27±9% vs 54±12%) groups concerning the control group [Figure 2]. Furthermore, theophylline did not inhibit the mast cell infiltration in comparison to the LPS-treated group (36±12% vs 28±11%) [Figure 2].

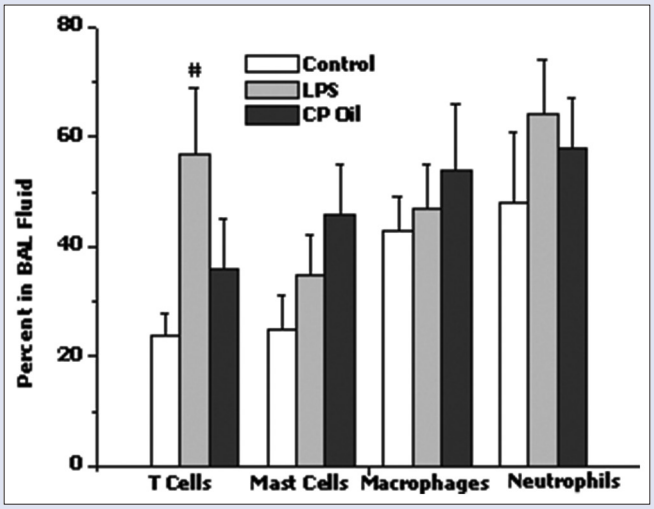

Figure 1: Percentage of various immune cells in the BAL fluid following one week of LPS exposure and CP oil treatment. Data represented as mean and SEM. (n ≥6 for each group from 4 animals from each group)

Effect on the infiltration of neutrophils

Neutrophils infiltrate into the lungs and release elastase which leads to degradation of connective tissues in the lungs and bronchi during the pathogenesis of emphysema and COPD.[23,24] Therefore, we assessed the level of neutrophils in BAL fluid of experimental animals and found no significant variations in the level of neutrophils across control (48±13%), LPS (64±10%), and CP oil (58±9%) groups following one week of treatment [Figure 1]. However, we observed a significant increase in the percentage of neutrophil infiltration in LPS-treated group (40±6% vs 68±6%) during long-term treatment and interestingly, a significant reduction in neutrophil infiltration was observed following treatment with theophylline (68±6% vs 32±9%; $P < 0.05$) and CP oil (68±6% vs 40±7%; $P < 0.05$) [Figure 2].

Effect on the infiltration of macrophages

Further, we analyzed the level of macrophages in the BAL fluid across all the experimental groups. Macrophages are usually present in the airway, alveoli, and lung interstitium, and may migrate into the lung space. It has been reported that the essential role of macrophages is to modulate acute and chronic inflammatory responses.[25] No significant increase in the level of macrophages was observed in the LPS-treated group from that of the control group during short-term LPS exposure (43±6% vs 47±8%). Moreover, there was no significant inhibition in macrophage level following CP oil administration (47±8% vs 54±12%) [Figure 1]. Further, we carried out a long-term study to understand the effect of CP oil during chronic inflammation settings and its effect on macrophages. There was no significant variation in the percentage of macrophages in the BAL fluid across control (22±8%), LPS (31±8%), CP oil (26±6%),

and theophylline (32±7%) groups [Figure 2], suggesting that CP oil and theophylline do not modulate the function of macrophages.

Effect on oxidative stress

Antioxidative properties of CP oil have been well established by many studies.[15,16] Therefore, we assessed the antioxidant potential of CP oil on short-term LPS exposure. Mean fluorescence intensity was taken as a parameter to determine the ROS generation potential across the control, LPS, CP oil, and theophylline groups. We observed a significant decrease in the level of ROS in the CP oil–treated group to that of the control group (5873 ± 1133 vs 2581 ± 1359; $P < 0.05$) [Figure 3]. Furthermore, we evaluated the antioxidant potential of CP oil on long-term LPS exposure and theophylline was taken as a positive control. We observed significant inhibition in ROS generation following treatment with CP oil from that of the LPS-treated group (20618 ± 1854 vs 5850 ± 1006; $P < 0.05$) [Figure 4]. Also, administration of theophylline reduced ROS generation significantly (20618 ± 1854 vs 5286 ± 2413; $P < 0.05$) [Figure 4].

GC-MS Analysis of *Celastrus paniculatus* seed oil

The graph obtained after the analysis of CP oil on GC-MS from Deve Herbes, India and CP oil from the local source were similar [Figure 5]. Linoleic acid (52.82%), palmitic acid (11.68%), supraene or squalene (2.45%), botulin (1.8%) were the major fractions from CP oil from Deve Herbes. Linoleic acid (39.63%) and palmitic acid (11.77%) are the major compounds found in the essential oil derived from CP oil from local sources as described in many studies.[7]

DISCUSSION

Many of the symptoms of COPD and asthma overlap, such as bronchitis, emphysema, asthmatic bronchitis, etc.[26,27] The major risk factors for COPD in the developed world are cigarette smoking, exposure to environmental pollutants, dust and fumes,[28] whereas exposure to biomass fuels and smoking are believed to be the major causes of COPD in the developing world.[29] The LPS present in environmental pollutants and cigarette smoke induces inflammation in the lung epithelium leading to life-threatening and non-reversible damage in the lungs.[30] Further, apart from smoking and environmental pollution, heredity may be a cause of COPD as non-smokers also have been found to develop COPD.[31]

Neutrophilic inflammation is considered to be a crucial feature of COPD. The toxic chemicals in cigarette smoke and environmental pollutants induce lung inflammation leading to infiltration of immune cells like neutrophils and macrophages.[32,33] Also, cigarette smoke, air pollutants, respiratory disorders and infections have the potential to produce oxidative stress and disturb the antioxidative balance in the lung milieu.[34,35] In a clinical trial, there were increased numbers of neutrophils and macrophages in bronchial biopsies from COPD patients.[32] These inflammatory cells, which are sources of ROS, proinflammatory cytokines or chemokines, and tissue-damaging enzymes are believed to be responsible for the development and progression of COPD.[35] Oxidants produced by cigarette smoke stimulate the alveolar macrophages to generate ROS and release several mediators which attract neutrophils and other inflammatory cells into the lungs. Neutrophils and macrophages are known to migrate in increased numbers into the lungs of cigarette smokers and can generate ROS via the NADPH oxidase system.[36] The lungs of smokers with airway obstruction have more neutrophils than smokers without airway obstruction.[36] Circulating neutrophils from cigarette smokers and patients with COPD, particularly during exacerbations of COPD, can release more $O_2^{\cdot-}$.[36] As the lung is continuously exposed to the outside environment, it is highly susceptible to injury mediated by oxidative stress. Further, ROS can activate various transcription factors and inflammatory responses in the lung.[37,38]

In the present study, we have used theophylline as a positive control to compare its effect on neutrophilic airway inflammation and infiltration to lungs. Low doses of theophylline have been found to exhibit anti-inflammatory effects and significantly reduce neutrophil numbers in COPD patients.[39] Further, theophylline has been shown to reduce other sputum inflammatory cells, interleukin-8, myeloperoxidase, lactoferrin and neutrophil chemotaxis.[39] Moreover, theophylline has been shown to inhibit the production of harmful reactive oxidants like superoxide by inhibiting of phosphodiesterase and increasing the level of cAMP.[40] Mast cells play a critical role in bronchial inflammation. Mast cells release vasoactive and spasmogenic mediators like adenosine,

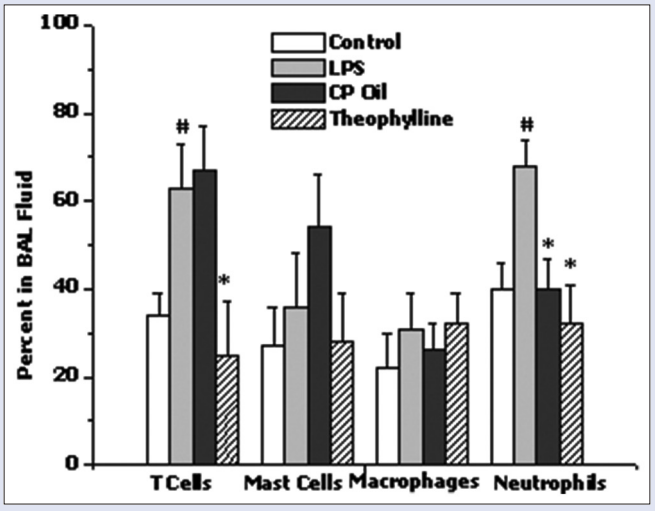

Figure 2: Percentage of various immune cells in the BAL fluid following one week LPS exposure and CP oil treatment. Data represented as mean and SEM. For #: $P < 0.05$ as compared to control group; for *: $P < 0.05$ as compared to LPS-treated group. ($n \geq 6$ for each group from 4 animals from each group)

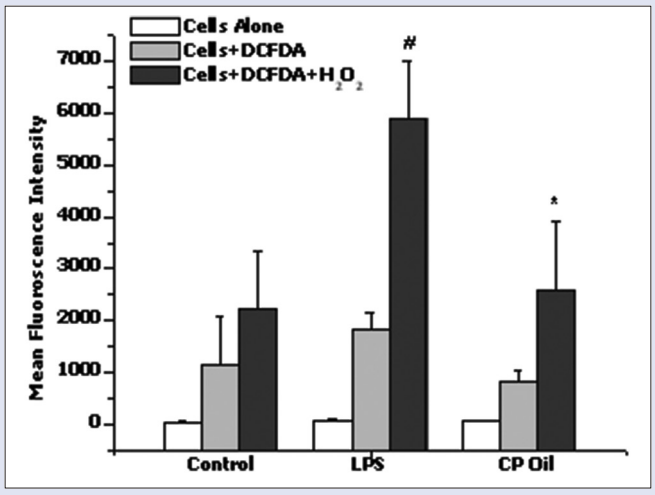

Figure 3: Effect of CP oil on generation of reactive oxygen species in BAL fluid on short-term LPS exposure. Data represented as mean and SEM. For #: $P < 0.05$ as compared to control group; for *: $P < 0.05$ as compared to LPS-treated group ($n \geq 10$ for each group from 4–6 animals from each group)

chemotactic factors, and enzymes. Theophylline has been found to inhibit adenosine, a mast cell mediator, thereby bringing about the reduction in the asthmatic episode.[41]

We demonstrated that CP oil significantly reduced the generation of ROS in BAL fluid. Also, various extracts from CP oil have been found to enhance cognitive function by exhibiting its antioxidant properties.[15,16] Though there is a detailed study on the pathophysiological mechanisms of COPD, there has been a little progress in developing a suitable pharmacological intervention for the disease.[24] Traditional and folk medicines are being used in India from ancient days for the treatment of chronic cough and respiratory inflammation. Therefore, traditional medicines with advanced scientific studies and formulations may offer better therapeutics for the treatment of COPD and respiratory inflammation. Also, there is a need to purify and isolate the various active principles in the CP oil to determine the mechanism of action of every entity for advanced therapeutics in future. Also, phytochemicals or active principles obtained from natural sources like CP oil may be evaluated for their protective effect against SARS-CoV-2 infections and mitigation of chronic cough.

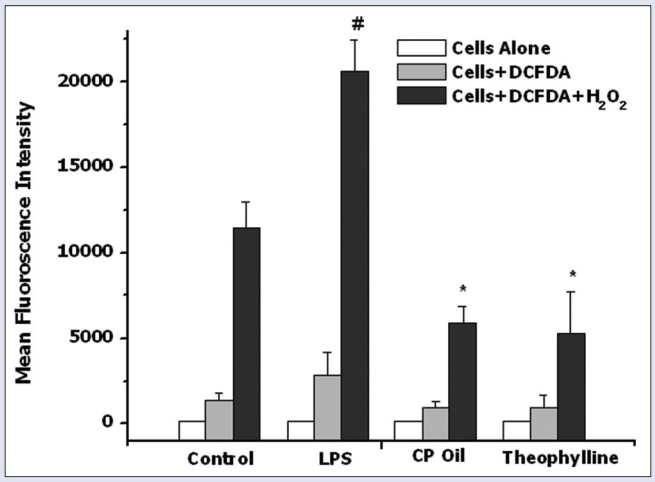

Figure 4: Effect of CP oil on generation of reactive oxygen species in BAL fluid on long-term LPS exposure. For #: $P < 0.05$ as compared to control group; for *: $P < 0.05$ as compared to the LPS-treated group ($n \geq 10$ for each group from 4–6 animals from each group)

CONCLUSION

From this study, we conclude that *Celastrus paniculatus* seed oil has significant antioxidant properties. Here, we have demonstrated that CP oil reduces oxidative stress by significantly inhibiting the generation of reactive oxygen species from immune cells in lipopolysaccharide-induced lung inflammation. CP oil does not modulate the infiltration of T cells, neutrophils, mast cells, and macrophages during short-term treatment. However, CP oil can modulate the infiltration of neutrophils following long-term treatment. Furthermore, reduction in generation of reactive oxygen species during long-term treatment seems to be through a reduction in infiltration of neutrophils. Further, we plan to conduct *in vitro* experiments using lung cancer cell lines to delineate the mechanism of action of CP oil and its active principles. Moreover, this study needs further corroborations by identifying the active principles from the CP oil and evaluating every constituent for its antioxidant properties for better therapeutics for chronic respiratory inflammation having good safety and efficacy profile.

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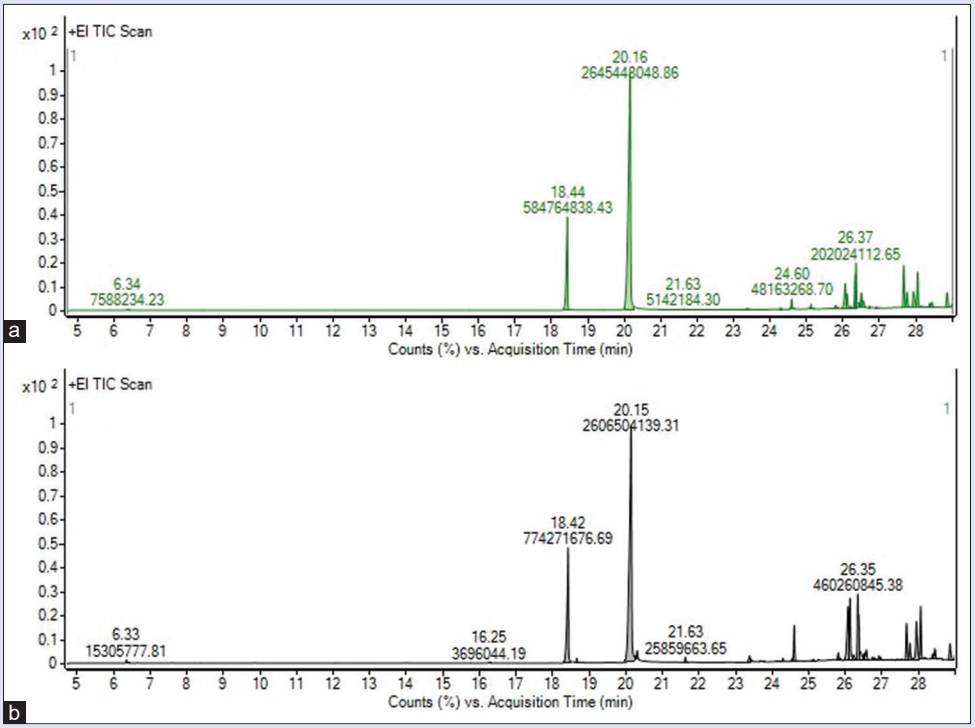

Figure 5: Analysis of CP oil in GC-MS. (a) GC-MS Graph of CP oil from Deve Herbs, India (b) GC-MS Graph of CP oil procured locally

Flow Cytometry Facility, IISc for support in flow cytometry studies and Mass Spectrometry Facility, Division of Biological Sciences, IISc for GC-MS analysis.

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Conflicts of interest

There are no conflicts of interest.

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