Cichorium intybus L. significantly alleviates cigarette smoke-induced acute lung injury by lowering NF-κB pathway activation and inflammatory mediators

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ABSTRACT

Background: Cigarette smoke (CS) is one of the primary causes of acute lung injury (ALI) via provoking pulmonary inflammation and oxidative stress. Despite substantial studies, no effective treatment for ALI is presently available.

Purpose: New prospective treatment options for ALI are required. Thus, this project was designed to investigate the in vivo and in vitro protective effects of 70% methanolic-aqueous crude extract of whole plant of Cichorium intybus (Ci.Mce) against CS-induced ALI.

Study design/methods: Initially, male Swiss albino mice were subjected to whole-body CS exposure for 10 continuous days to prepare CS-induced ALI models. Normal saline (10 mL/kg), Ci.Mce (100, 200, 300 mg/kg), and Dexamethasone (1 mg/kg) were orally administered to respective animal groups 1 h prior to CS-exposure. 24 hrs after the last CS-exposure, BALF and lungs were harvested to study the key characteristics of ALI. Next, HPLC analysis was done to explore the phytoconstituents.

Results: Ci.Mce exhibited significant reductions in lung macrophage and neutrophil infiltration, lung weight coefficient, and albumin exudation. Additionally, it effectively ameliorated lung histopathological alterations and hypoxemia. Notably, Ci.Mce exerted inhibitory effects on the excessive generation of IL-6, IL-1β, and KC in both CS-induced ALI murine models and CSE-stimulated RAW 264.7 macrophages. Noteworthy benefits included the attenuation of oxidative stress induced by CS, evidenced by decreased levels of MDA, TOS, and MPO, alongside enhanced TAC production. Furthermore, Ci.Mce demonstrated a marked reduction in CS-induced NF-κB expression, both in vivo and in vitro.
Conclusion: Consequently, Cichorium intybus could be a therapeutic option for CS-induced ALI due to its ability to suppress inflammatory reactions, mitigate oxidative stress, and quell NF-κB p65 activation.

List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ALI</td>
<td>Acute lung injury</td>
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<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<td>Ci.Mce</td>
<td>Methanolic crude extract of Cichorium intybus</td>
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<tr>
<td>CS</td>
<td>Cigarette smoke</td>
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<tr>
<td>CSE</td>
<td>Cigarette smoke extract</td>
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<tr>
<td>Dex</td>
<td>Dexamethasone</td>
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<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte chemoattractant</td>
</tr>
<tr>
<td>LWC</td>
<td>Lung weight coefficient</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MTT</td>
<td>Methylthiazol-tetrazolium test</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NS</td>
<td>Normal saline</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>TAC</td>
<td>Total antioxidant capacity</td>
</tr>
<tr>
<td>TFC</td>
<td>Total flavonoid contents</td>
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<tr>
<td>TOS</td>
<td>Total oxidative stress</td>
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<tr>
<td>TPC</td>
<td>Total phenolic contents</td>
</tr>
<tr>
<td>WBCs</td>
<td>White blood cells</td>
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</table>

1. Introduction

Serious form of acute lung injury (ALI) known as acute respiratory distress syndrome (ARDS) is characterized by pulmonary infiltrates, neutrophil-derived inflammation, hypoxemia and edema [1]. Direct causes of ALI/ARDS include pneumonia, aspiration of stomach material, inhalation damage and trauma [2], indirect causes include septic shock, blood transfusion, lipid embolism, burns, and hypovolemia [3,4].

Chronic or intermittent exposure to cigarette smoke (CS) is a one of the major contributor to ALI/ARDS risk factors and vulnerability [5,6]. More than 5000 different molecules combine to form CS all of which contribute to oxidative stress [7]. Exposure to CS through inhalation impairs immune function [8], alter alveolar oxidative stress [9,10], increases macrophage and neutrophil incursion in the alveoli and lungs [11,12], increases endothelial and pulmonary permeability [13,14] and stimulates the formation of pro-inflammatory cytokines and chemokines (IL-1β, TNF-α, IL-6, IL-8) [15], ROS and proteases [16], that causes alveoli and lungs are to be demolished [17]. Activated alveolar macrophages also secrete an increase in pro-inflammatory cytokines which in turn draw in an increase in neutrophils [18], exacerbating the inflammatory response and worsening ALI/ARDS [19]. The pulmonary inflammation in animal models exposed to CS is exacerbated [20,21]. Since the immune system, metabolic pathway, and lung injury are the same in mice and humans, the therapeutic evaluation of ALI and ARDS can assessed by the use of animal models.

NF-κB, a heterodimer mostly made up of P50 and P65, regulates inflammation that is redox sensitive. Due to the action of the inhibitory protein IκB, in particular IκBα, NF-κB remains dormant in the cytoplasm of unstimulated cells [22,23]. In the nucleus, the released NF-κB complex then induces the transcription of various cytokines as well as other inflammatory mediators (nitric oxide and PGD2/E2), which may contribute to CS-induced airway inflammation [24,25]. Inflammatory cytokines and oxidative stress and, all of which are present in or created by tobacco smoke, have an impact on NF-κB signalling. Evidence suggests that suppressing NF-κB also decreased pulmonary edema and neutrophil influx, two symptoms of ALI that are characterised by oxidative stress and the generation of pro-inflammatory cytokines [26,27].

Many communities still rely on indigenous medicinal plants to supply their basic health care requirements. Cichorium intybus L.
often known as “Kasni,” grows wild in Pakistan’s Punjab and NWFP regions [28]. All portions of this plant have high therapeutic potential owing to the presence of alkaloids, mucilage, saponins, coumarins, unsaturated sterols, vitamins, tannins sesquiterpene lactones, flavonoids, chlorophyll pigments, and inulin [29]. Cichorium intybus possesses immune-boosting [30], antihypertensive [31], antimicrobial [32], anthelmintic [33,34], antimalarial [35], hepatoprotective [36], antidiabetic [37,38], gastro protective [39], analgesic [40], anti-neurotoxic [41], anti-tumor [42], antiallergic [43], anti-inflammatory [44] and antioxidant [45–51] properties. The antioxidant and anti-inflammatory properties of Cichorium intybus prompted us to investigate the protective effect of Cichorium intybus extract against CS-induced ALI mouse models.

2. Materials and methods

2.1. Chemical

Research-grade materials were used during the whole experiment. Most chemicals, involving DPPH radical, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, ketamine, xylazine, formalin (10 %), dexamethasone, methanol, acetonitrile (ACN), 0.9 % normal saline (NS) and Wright-Giemsa stain, are employed. Clark and Lubs solution, ferrous ion with o-dianisidine, acetate buffer, sodium carbonate, gallic acid, trichloroacetic acid (20 %), n-butanol, trichloroacetic acid (0.67 %) and n-butanol are also used. We bought MPO and MDA kits from Jiancheng Bioengineering Institute in Nanjing (Nanjing, China) while ELISA kits of Keratinocyte chemoattractant (KC), and IL-1β and IL-6 were purchased from 4A Biotech Co. Ltd. (Beijing, China) and Multisciences Biotech Ltd. (Hangzhou, China) respectively.

2.2. Plant product

Fresh whole plant of Cichorium intybus were collected during flowing and fruiting season (in June–October 2021) from district Bahawalpur, Pakistan, dried under shade and sliced without washing. Following a botanist’s confirmation of macroscopic and microscopic anomalies (voucher number CI-WP-08-21-203), dried plant specimens were sent to the herbaria of the pharmacology research lab in the Pharmacology Department at the Islamia University of Bahawalpur (IUB), Pakistan.

2.3. Preparation of crude extract

Cichorium intybus dry plant was hand-picked to eliminate impurities and processed into a coarse powder (#40). The crushed plant material was macerated in 70 % aqueous methanol (70 % Aq.MeOH) for 72 h. Muslin cloth is used to filter away leftovers, and Whatman grade 1 filter paper is used to filter the liquid. To increase extract yield, residue was re-soaked in 70 % Aq.MeOH. Filtrate was saved in amber jars. This methanolic crude extract of Cichorium intybus entitled as Ci.Mce was stored at 4 ◦C after being thickened in a rotary evaporator (Heidolph, Germany) at 760 mmHg, 40–45 ◦C and 90–120 rpm. Using Equation 1, the percentage yield of the Ci.Mce was calculated to be 12 % [52].

\[
\text{Percentage yield} = \frac{\text{Theoretical yield (g)}}{\text{Actual yield (g)}} \times 100
\]

\[
\text{Percentage yield of Ci.Mce} = \frac{180g}{1500g} \times 100 = 12\%
\]

Equation 1: Equation for the percentage yield calculation.

2.4. HPLC analysis

HPLC analysis of Cichorium intybus was performed as previously reported method [53]. A UV/visible detector set at 280 nm were then used to analyze the retention duration, peak areas and identifying the substances.

2.5. Analysis of total phenolic and flavonoid contents

The TPC [54] and TFC [55] of a sample extract were determined using slightly modified the Folin-Ciocalteu reagent (FCR) and Aluminium chloride (AlCl3) method respectively. Gallic acid and Quercetin were used as standard to measure TPC and TFC respectively. At 760 nm and 415 nm, the absorbance was measured for TPC and TFC respectively. The results were given in milligrams of standard equivalent per grams of dry extract (mg. Eq.g-1 DE).

2.6. DPPH assay

Extracts’ ability to scavenge DPPH free radicals was evaluated using a technique with minimal adjustments that utilized ascorbic acid as a reference [56]. The absorbance was measured at 517 nm.
2.7. ABTS assay

To assess plant sample ability to quench free radicals, the ABTS radical cation decolorization analysis was conducted according to previously described method [43]. The outcomes are stated in milligrams of Trolox equivalent per grams of dry extract.

2.8. Procedure of dose preparation

The necessary quantity of Ci.Mce was determined with the use of an analytical scale and then dissolved in normal saline. 10 mL/kg was used as standard dosage criteria. The amount given to each mouse was calculated and adjusted according to body weight then given by mouth through a feeding tube.

2.9. Ethical considerations in the handling of mice

After receiving clearance from the Department of Pharmacology, Faculty of Pharmacy, IUB, Pakistan’s Animal Research Ethics Committee (PAEC/2022/21), male Swiss albino mice (20–30 ± 2 g; 6–7 weeks old) free of pathogens were kept in ventilated wooden cages. Every animal was kept in the same conditions (24°C ± 2) 40–60% humidity, 12 h of light and 12 h of darkness and unrestricted access to food and water. Regular assessments of their weight and general health were performed. They had some time to adjust to their surroundings before the experiment. Each of the six specified experimental mouse groups, which each included five mice, was employed and accurately labeled: 1) healthy, 2) CS-exposed, 3) Dexa (1 mg/kg), 4) 100 mg/kg Ci.Mce, 5) 200 mg/kg Ci.Mce, and 6) 300 mg/kg Ci.Mce.

2.10. Preparation of CS-induced acute lung injury models

The CS-induced ALI models were produced using the formerly outlined method [57]. Dexa and Ci.Mce were administered orally to the appropriate groups in doses of 100, 200 and 300 mg/kg each. Except for the control/healthy group, all the groups were placed in a polycarbonate chamber measuring 65 × 50 × 45 cm, linked to an exhaust pipe and exposed to 3R4F research grade cigarette smoke for an hour [58]. One cigarette is given to mice every 6 min or around ten cigarettes each day. The procedure was carried out continually for ten days. Twenty-four hours following the last CS exposure, oxygen saturation of all mice was assessed and collection of blood samples was done. Each of them underwent cervical dislocation to assemble BALF for the assessment of inflammatory cell enumeration and lung tissues for histopathology.

2.11. Assessment of oxygen saturation

Twenty-four hours subsequent to the final exposure to CS (cigarette smoke), the moor VMS-OXY™ monitor sourced from Moor Instruments in the United Kingdom was utilized [59]. This monitoring instrument was engaged to determine the oxygen saturation percentage (SO2) in all mice. It evaluates both the concentration of oxygenated and deoxygenated hemoglobin, along with the oxygen saturation (measured as a percentage), within the microcirculatory system. This evaluation takes place within the specific wavelength range spanning from 500 to 650 nm.

2.12. Blood collection

Before sample collection, anaesthetize the animals. Connect a suitable sized needle to a syringe and insert it at a 30–40° angle into the diaphragm of animals. Pull the plunger back to create a vacuum in the syringe then move the needle forward until blood is seen. After collecting the blood, quickly euthanize the animals via cervical dislocation. Immediately pour the blood into EDTA tubes to avoid clotting and send it for testing of neutrophils and WBCs count to lab.

2.13. BALF acquisition and inflammatory cell counting

BALF is a more accurate sample because to its closeness to the injury and capacity to monitor damage progression [60]. All the mice were killed and the thoracic chamber and trachea were surgically exposed. To stop fluid from getting into the right lungs, a thread was used to ligate them. A blunt needle was used to inject 0.4 mL of pre-cooled, sterile NS into the left lungs and the withdrawal was carried out carefully to prevent shearing forces. Three lavages were done and the samples were put on ice. Inflammatory cells like as neutrophils, lymphocytes and macrophages were counted in a Neubauer chamber (LabOptik, Friedrichsdorf, Germany). The extra BALF was immediately centrifuged (1000×g; 4°C) for around 10 min. The cell pellet was spread on glass slides to create the smears and the collected supernatant portion was preserved at ~80°C [61]. Two experts used structural criteria of inflammatory cells to count 200 cells (10⁴/mL BALF) after Wright-Giemsa staining of the generated smears.

2.14. Lung weight coefficient (LWC)

Separated lungs were weighed right away after removal in order to calculate the lung-weight ratio which is a sign of pulmonary edema. After aspirating the surface blood of the lung tissues, LWC was calculated by dividing the weight of each animals individual lung by its total body weight [62]. Then the lungs of every mouse were carried out for pathological examination.
2.15. Albumin assay

Albumin determination kits were employed to analyze the albumin concentration within BALF supernatants. The assay method employed the bromocresol green technique. Initially, all necessary reagents, including the albumin standard and bromocresol green solution, were prepared in accordance with the manufacturer’s guidelines. Standard tubes received albumin standard (0.02 mL) and bromocresol green solution (5 mL), while sample tubes contained BALF supernatants (0.02 mL) and bromocresol green solution (5 mL). Thorough mixing was performed for all tubes, which were then left at room temperature for 10 min. Subsequently, optical density measurements were taken using a spectrophotometer at 628 nm. The ratio of albumin concentrations derived from BALF not only reflects the levels of effused albumin but also provides insights into pulmonary microvascular permeability [63].

2.16. Lung histopathology

The right lower lobe of mice lungs was preserved in a 10 % formalin solution at ambient temperature and the lobe was then fixed in paraffin for long-term preservation. As a result, marked histology cassettes containing attached lung tissues were cleansed with running tap water for around two to 3 h and then dehydrated using graded alcohols (70–100%) and xylene for varied amounts of time. Then, using a stainless steel mould with a stainless steel plastic ring around the edge, molten paraffin was injected into the lung tissues and allowed to set on a cold plate. The primary intrapulmonary bronchus was completely visible when the lungs were sliced (4 μm) using a tissue slicer and placed on a glass surface or slide. The slide was dewaxed using xylene, alcohol and stained using Hematoxylin and Eosin (H&E). The sectioned tissue was mounted on glass slides using Canada balsam. After that, images were captured with a camera affixed to a microscope at 20X, 40X. Alveolar edema, degree of injury and inflammatory cell infiltration were all graded by pathologists using a five-point scale [62]. Each lung component received a score in at least three different categories. From 0 to 5, the degree of lung injury was rated and the average grade across all factors was calculated.

2.17. Analysis of cytokines and chemokines via ELISA

The supernatant of BALF was used to measure the proinflammatory cytokines (IL-1β, IL-6) and chemokines (KC) by using commercially available ELISA kits as per instructions of the manufacturer. Optical density quantification was done at 450 nm and the standard curves were used to express the cytokine levels.

2.18. Estimation of total oxidative stress (TOS) and total antioxidant capacity (TAC)

To perform TOS and TAC analysis, commercially available kits were used as per instructions of manufacturer. The semi-automatic biochemistry analyzer was used for the spectrophotometric analysis (Biosystem BTS-330).

2.19. Assessment of malondialdehyde (MDA) and myeloperoxidase (MPO) activities

To evaluate the evaluation of MPO levels and MDA activity, segments of left lung tissue weighing 50 mg were rinsed, subsequently homogenized and centrifuged at 4500g for duration of 10 min. The collected supernatant of BALF was analyzed at 460 nm using MPO and MDA determination kits in accordance with protocols developed by the manufacturer.

2.20. Extract of cigarette smoke (CSE) preparation

A previously developed method was used for CSE preparation [62]. As precisely describing, CS produced from ten research-cigarettes was passed over 100 mL of PBS (each being lit for 5 min) through a vacuum pump. At 80 °C, prepared CSE was kept.

2.21. Cell culture and cell viability assay

RPMI-1640 medium was used for in vitro investigation with FBS (10 %), penicillin (100 U/mL) and streptomycin (100 μg/mL) to cultivate RAW 264.7 macrophage. First, the cytotoxicity of plant extract alone up to 0–200 μM, CSE alone up to 1–10 %, and combination of CSE (1–10 %) and plant extract (0–200 μM) on RAW 264.7 macrophage were evaluated via methylthiazol-tetrazolium test (MTT). Briefly, in 96-well plates RAW 264.7 macrophage (concentration of 4 × 10^5 cells/mL) were cultured for 12 h and then treated with plant extract (0–200 μM) at 37 °C for 1 h. Then, RAW 264.7 macrophages were then treated for 24 h with CSE (1–10 %) before mixing with MTT (5 mg/mL) for yet another 4 h. Absorbance was determined at 570 nm after replacement of each plate’s supernatant with 200 μL of DMSO. MTT assay revealed that plant extract up to 100 μM, CSE up to (4 %), and a combination of CSE (up to 4 %) and plant extract (up to 100 μM) were not harmful for RAW 264.7 macrophage. Thus, the dosages of 10 and 100 μM of plant extract and CSE 4 % were chosen for exposure to RAW 264.7 macrophages.

2.22. In vitro cytokines assay

For an in vitro analysis of cytokine levels in RAW 264.7, 12-well plates’ culture media was substituted with RPMI-1640 medium
(serum-free) once macrophage cell lines had acclimated to it. After 12 h, cell lines were exposed to plant extract at the concentration of 10–100 μM for 1 hr then subjected for another 24 h with CSE (4 %). After treating with CSE, levels of IL-1β, IL-6, and KC was evaluated by ELISA kits.

2.23. Analysis of p65-NF-κB

For investigating p65-NF-κB, an ELISA kit (Ab176648) from Abcam, was utilized. Typically, tissue lysates were made by centrifuging preserved lungs at 18,000 × g for 20 min to recover the supernatants after they had been diced and carefully washed in PBS to remove blood. A plate reader set to 450 nm was used to measure absorbance.

2.24. ELISA measurement of p65-NF-κB

Ab176648, an ELISA kit from Abcam, was used to investigate p65-NF-κB. Typically, tissue lysates were made by centrifuging preserved lungs at 18,000 × g for 20 min at 4 °C to collect the supernatants after they had been diced and carefully washed in PBS to remove blood. A plate reader set to 450 nm was used to measure absorbance.

2.25. Statistics

All the data was calculated as mean ± SD (Standard deviation) and the homogeneity in animal’s weight distribution was calculated by applying the coefficient of variation. The test was conducted using the Graph Pad Prism programme, and the significant values were #p < 0.05, ##p < 0.01 compared the control group, and * p < 0.05 and **p < 0.01 vs. the model group.

3. Results

3.1. HPLC assessment of Ci.Mce

HPLC is used in analytical chemistry to isolate, characterize and quantify sample parts. The chromatographic standardization using isocratic HPLC revealed that the most active components in the Ci.Mce were phenolic acids such as m-coumeric acid, p-coumeric acid, vanillic acid, syringic acid, gallic acid, ferulic acid and chlorogenic acid along with flavonoids including kaempferol and quercetin (Table 1). Chromatogram demonstrating the presence of kaempferol (Fig. 1) phenolic acid and quercetin (Fig. 2).

3.2. Effect of Ci.Mce on TFC and TPC

Phenolic chemicals are significant plant components with redox characteristics that confer antioxidant action because their hydroxyl groups facilitate the scavenging of free radicals. One of the main classes of phenolic chemicals are flavonoids, which have a wide range of chemical and biological functions, particularly radical scavenging. The phenolic content expressed as gallic acid equivalent per gram (GAE/g) in the Ci.Mce was found 152.96 ± 6.67 mg GAE/g, which is comparable to the reference standard (157.68 ± 3.27) as shown in Table 2. Similarly, total flavonoid concentration of Ci.Mce is 58.802 ± 2.79 mg. Qu.Eq/g (expressed as Quercetin equivalents) which is comparable to Quercetin (56.82 ± 5.32) (Table 2).

3.3. Antioxidant capacity of Ci.Mce by ABTS assay

The ability of Ci.Mce to fight ABTS + radicals as an antioxidant has been compared to that of Trolox, which is used as a standard. To produce a stable radical cation of ABTS, potassium per sulphate was utilized. The antioxidant potential was determined by examining decolorization following the addition of the plant extract and after the absorbance had stabilized. The ABTS test yielded Ci.Mce values of 130.88 ± 4.26 mg, TEAC/g DW (Table 2).

Table 1: HPLC-based qualitative and quantitative assessment of phyto-constituents.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>RT (min)</th>
<th>Area (mV.s)</th>
<th>Quantity(ppm)</th>
<th>Possible Compound</th>
<th>Molecular formula</th>
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<tr>
<td>1</td>
<td>2.8553</td>
<td>330.493</td>
<td>17.52</td>
<td>Quercitin</td>
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<tr>
<td>2</td>
<td>4.720</td>
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<td>806.806</td>
<td>20.17</td>
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<td>1038.600</td>
<td>446.34</td>
<td>Kaempferol</td>
<td>C15H10O6</td>
</tr>
</tbody>
</table>


3.4. Effect of Ci.Mce on DPPH activity

Plant antioxidants affect DPPH solution color. When the antioxidants eliminate the radical through proton transfer, absorbance shifts from indigo to light golden. Antioxidants may scavenge DPPH radicals by donating hydrogen. In DPPH tests, Ci.Mce scavenged free radicals well. As concentration increases, absorbance drops and the extract percentage inhibition grows, as indicated in Table 3. According to these results, Ci.Mce has shown a considerable antioxidant effect at the tested concentrations. Ascorbic acid and Ci.Mce have the highest antioxidant activity at the 0.5 mg/mL (86.13 % and 81.02 % DPPH inhibition, respectively) with IC\textsubscript{50} values of 76 μg/
mL and 81 μg/mL.

3.5. Effect of Ci.Mce on CS-induced hypoxemia

Using a moor VMS-OXYTM monitor, the SO$_2$ levels of the mice were measured 24 h after their last exposure to CS. As demonstrated in Fig. 3, healthy animals on average had a 98% SO$_2$ levels while CS-exposed mice developed hypoxemia (lower SO$_2$ up to 87 %) compared to Dexa and Ci.Mce-treated animals. Administration of Ci.Mce (100 mg/kg, 200 mg/kg, and 300 mg/kg) improved the SO$_2$ levels dose-dependently. The 300 mg/kg dose notably enhanced oxygen saturation to 97% (p < 0.01), comparable to healthy animals and Dexa (1 mg/kg) treated animals. Ci.Mce shows promise in mitigating CS-induced hypoxemia, particularly at the 300 mg/kg dose.

3.6. Effect of Ci.Mce on WBCs and neutrophil count

After collecting cardiac blood from mice, it was transferred to EDTA tubes to prevent clotting. Subsequently, the levels of white blood cells (WBCs) and neutrophils were measured. In healthy mice, the average cellular concentration of white blood cells (WBC) and neutrophils was $7.5 \times 10^3/\mu l$ and $3.1 \times 10^3/\mu l$, respectively. Following exposure to cigarette smoke, these levels increased significantly to $17.2 \times 10^3/\mu l$ for WBC and $4.4 \times 10^3/\mu l$ for neutrophils, confirming the occurrence of systemic inflammation following exposure to cigarette smoke. However, when mice were administered Ci.Mce at doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg, the average cellular concentration of WBC and neutrophils returned to normal levels dose-dependently, comparable to those in the control group not exposed to cigarette smoke, as shown in Fig. 4 (A, B). Notably, Ci.Mce exhibited a dose-dependent effect, with the dosage of 300 mg/kg being particularly effective in lowering WBCs ($9.9 \times 10^3/\mu l$) and neutrophils ($3.5 \times 10^3/\mu l$) compared to both 100 and 200 mg/kg (p < 0.01). This outcome underscores the efficacy of Ci.Mce in mitigating the cellular concentration disruptions induced by cigarette smoke exposure.

3.7. Effect of Ci.Mce on CS-induced lung edema and permeability

LWC and lung albumin contents were used to measure lung edema and lung permeability respectively. CS not only effectively induces oxidative stress but also increases microvascular permeability and edema, both of which lead to severe pulmonary inflammation. The LWC and lung albumin contents were significantly elevated in the model group contrast to the control group (p < 0.01). Comparatively to the model group, the Ci.Mce significantly and dose-dependently lessened LWC (p < 0.01) (Fig. 5A) and albumin contents (p < 0.01) (Fig. 5B).

3.8. Effect of Ci.Mce on CS-induced inflammatory cell infiltration in BALF

In the pathogenesis of ALI, cellular infiltration into the lungs is a significant occurrence. Inflammatory cell count, including neutrophils, lymphocytes and macrophages, was checked in the BLAF collected from the sacrificed animals. Total number of inflammatory cells, neutrophils, lymphocytes and macrophages count was analyzed by using Wright–Giemsa staining method. Comparatively to the group exposed to fresh air, CS exposure significantly increased the number of total inflammatory cells, neutrophils, macrophages, and lymphocytes (p < 0.01) as shown in Fig. 6. However, the CS-induced increases in the number of total inflammatory cells, neutrophils, and macrophages were substantially and dose-dependently suppressed by Ci.Mce administration (p < 0.01). This shows that Ci.Mce may be able to reduce CS-induced ALI.

Fig. 3. Ci.Mce reduces the hypoxemia caused by CS. A pulse oximeter was used to measure SO$_2$ levels 24 h following the last CS exposure. Ci.Mce raised SO$_2$ and reduced CS-caused hypoxemia. *p < 0.05 and **p < 0.01 are comparisons to the CS-exposed groups while ***p < 0.01 is comparison to the healthy group. (n = 5 in each group).
3.9. Effect of Ci.Mce on CS-induced lung histopathological alterations

To investigate Ci.Mce ability to protect against CS-induced lung histopathologic alterations, H&E staining was used. The control group had normal lung histology but the CS-exposed group had scattered alveoli, alveolar wall inflammation, pulmonary edema and an increase in inflammatory cells in the alveolar gaps (Fig. 7A). Ci.Mce or Dexa considerably improved histopathological alterations. Ci.Mce decreased neutrophil levels and enhanced lung airflow in mice. Histopathology shows dose-related improvement. Two blinded pulmonary pathology specialists evaluated the mean pathological score to determine the degree of injury, inflammatory cell infiltration and fluid retention. The use of Ci.Mce ($p < 0.01$) and Dexa ($p < 0.01$) reduced the average pathological grade in CS-exposed mice. The pathological grade in the CS group was higher ($p < 0.01$) than in the healthy group. From histology and pathology data, Ci.Mce lowers CS severity (Fig. 7B).

3.10. Effect of Ci.Mce on CS-induced release of pro-inflammatory cytokines and chemokines

Respective ELISA kits were used to check the levels of proinflammatory cytokines (IL-6, IL-1β) and chemokines (KC) as shown in Fig. 8 (A-C). Notable rise in the levels of IL-6, KC, and IL-1β was noted in the BALF of model group as compared to the control and treatment groups. However, in contrast to the model group, the Dex and Ci.Mce groups showed a dose-dependent reduction in the expression of these markers ($p < 0.01$). These results imply that Ci.Mce protected the lungs from CS-induced ALI by reducing IL-1β, IL-
3.11. Effect of Ci.Mce on CS-mediated TOS production

Oxidative stress results in severe lung injury; thus, the TOS in the BALF of mice exposed to CS was assessed. CS exposure substantially increased the TOS in the CS-mediated group in contrast to the control group ($p < 0.01$). The Dexa (1 mg/kg) and Ci.Mce (300 mg/kg) treatment remarkably and dose-dependently decreased CS-mediated TOS over-expression (Fig. 9). There was a significant difference ($p < 0.01$) between the Ci.Mce and Dexa groups and the model group in terms of oxidative stress reduction.

3.12. Effect of Ci.Mce on CS-mediated TAC production

Natural antioxidants are overwhelmed by the oxidative stress generated by the CS, resulting in increased lung damage. The TAC test was used to evaluate the antioxidant properties of Ci.Mce in mice exposed to CS. TAC was significantly lowered ($p < 0.01$) in the group exposed to CS compared to the control group. In addition, there were statistically significant differences between the Dexa ($p < 0.001$) and Ci.Mce ($p < 0.01$) groups and the model group (Fig. 10). It has been shown that Ci.Mce is proven to have an antioxidant effect opposed to oxidative stress in CS-mediated ALI.

3.13. Effect of Ci.Mce on CS-mediated MPO and MDA activity in BALF

Neutrophils infiltration and lung injury stimulate the release of MPO and a high accumulation of neutrophils results in exaggerated MPO actions. The lungs of the model group showed a significant MPO activity as compared to the control and treatment groups ($p < 0.01$) (Fig. 11 A). MDA, a lipid peroxidation indicator is created when reactive oxygen species causes oxidative degradation of cellular membranes [64]. Fig. 11 B shows that the MDA grade in the CS-exposed group was substantially elevated ($p < 0.01$) than in the air-exposed group. When compared to the CS-exposed group, both the Dexa and Ci.Mce treated groups showed considerably lower levels. Ci.Mce decreases the risk of lipid peroxidation in a dose-dependent way. Ci.Mce (300 mg/kg) substantially decreased MDA levels ($p < 0.01$) demonstrating that it possesses antioxidant activity against lipid peroxidation in CS-induced ALI. ROS initiate oxidative membrane degradation which results in MDA formation.

3.14. Effect of Ci.Mce on CS-stimulated release of pro-inflammatory cytokines and chemokines in RAW 264.7 macrophages

To validate the in vivo findings, it was further explored if the antioxidant and anti-inflammatory effect of Ci.Mce could prevent release of cytokines from CSE (4 %)-stimulated RAW 264.7 macrophages. According to the MTT test, Ci.Mce at doses up to 100 μM was safe for RAW 264.7 macrophages when combined with CSE (4 %). The effects of Ci.Mce on cytokine secretion were studied using ELISA. As expected, CSE (4 %) exposure resulted in noticeably high secretion of IL-6, IL-1β and KC ($p < 0.001$) while Ci.Mce (10 and 100 μM) dose dependently and significantly decreased expression of IL-6, IL-1β and KC from CSE-stimulated RAW 264.7 macrophages ($p < 0.001$) (Fig. 12 A–C). Therefore derived in vitro findings (Fig. 12) were in line with in vivo results (Fig. 8).
(caption on next page)
3.15. Effect of Ci.Mce on CSE-induced p65-NF-κB activation both in vitro and in vivo

Investigating how Ci.Mce lessens CS-induced ALI was done using ELISA. Because NF-κB is required for the activation of pro-inflammatory mediators, alterations in pulmonary vascular permeability, macrophages and neutrophil infiltration, and oxidative stress, this study concentrated on the effect of Ci.Mce on CS-induced NF-κB pathway activation. As seen in Fig. 13, lung tissues exposed to CS had considerably higher expression levels of p65-NF-κB than those treated with a vehicle. As a result, Ci.Mce administration at doses of 100, 200, and 300 mg/kg in CS-exposed ALI mice (Fig. 13A) and at doses of 10 and 100 μM CSE-stimulated RAW 264.7 macrophages (Fig. 13B) dose dependently and significantly decreased the amount of p65-NF-κB activation (*p < 0.01) respectively. By blocking the p65-NF-κB signalling pathway, Ci.Mce defended against CS-induced ALI at both in vitro and in vivo levels.

4. Discussion

ALI is a leading cause of death and disability among the severely unwell. The collapse of lung tissues due to protein-rich fluids exuding into the alveoli leads to the progression of acute lung injury (ALI) [65]. Smoking is one of the major causes of ALI/ARDS since one puff of a cigarette contains a number of organic chemicals, dangerous oxidants and free radicals (such as nitric oxide, superoxide,
Fig. 9. Ci.Mce lowered the CS-mediated TOS. *$p < 0.05$ and **$p < 0.01$ are comparisons to the CS-exposed groups while ***$p < 0.01$ is comparison to the healthy group. ($n = 5$ in each group).

Fig. 10. Ci.Mce strengthened TAC in CS-induced ALI. *$p < 0.05$ and **$p < 0.01$ are comparisons to the CS-exposed groups while ***$p < 0.01$ is comparison to the healthy group. ($n = 5$ in each group).

Fig. 11. Effect of Ci.Mce on pulmonary MPO activity (A) and MDA levels (B). *$p < 0.05$ and **$p < 0.01$ are comparisons to the CS-exposed groups while ***$p < 0.01$ is comparison to the healthy group. ($n = 5$ in each group).
Fig. 12. Ci.Mce altered the expression of CS-stimulated cytokines in RAW 264.7 macrophages. The harvested supernatant from pretreated RAW 264.7 macrophages was exposed to CSE to determine level of IL-6 (A), IL-1β (B), and KC (C). **p < 0.01 versus control group; ***p < 0.01 as opposed to model or CS group.

Fig. 13. In response to CS stimulation, Ci.Mce prevented the p65-NF-κB signalling pathway in the lungs (A) and RAW 264.7 macrophages (B) from being activated. *p < 0.05 and **p < 0.01 are comparisons to the CS-exposed groups while #p < 0.01 is comparison to the healthy group. (n = 5 in each group).
hydro-peroxides and hydroxyl). While supportive management continues to be the basis of treatment, there is presently no viable medicine available to treat ALI/ARDS despite significant advancements in clinical diagnosis and care [59]. Several research studies have been carried out to better understand the process of CS-mediated pulmonary damage and evaluate the effectiveness of prospective therapeutic drugs. However, many efficient agents have been found. As a consequence, there is an essential need for efficient and secure treatments to lessen the CS-related pulmonary inflammation [62].

Thousands of free radicals are present in a whiff of CS [66] including nitric oxide superoxide, hydroxyl and hydro peroxide [58]. CS includes organic compounds, oxidants and free radicals. More fatalities and diseases are caused by CS than any other reason (involving alcohol intake, HIV, accidents and illicit drug usage). A smoker’s life expectancy is 14 years lower than a nonsmoker’s [67]. Over 3 million people, predominantly in the industrialized world die each year from smoking-related causes. ROS damage increases pulmonary epithelium and endothelial permeability allowing macrophages and neutrophils to enter the lungs [68]. Chemokines and pro-inflammatory cytokines generated by alveolar macrophages worsen ALI/ARDS [5]. Preclinical and clinical research indicates CS harmful impact on human health. Because the metabolic system, immunological processes and lung damage in ALI are the same in mice and humans. Mice are an excellent model for evaluating therapies [69]. In the last decade, huge investigations have been conducted to prove the antioxidant and polyphenols biological characteristics. Medicinal plants contains quinones, curcuminoids, tannins, flavonoids, coumarins, and phenolic acids [70]. Quercetin and Kaempferol reduce lung deterioration and edema to avoid ALI in mice [71]. HPLC revealed that Cichorium intybus contains polyphenols, including phenolic acids and flavonoids [53]. Antioxidant ability of Cichorium intybus to quench ABTS β radicals has been evaluated. The total phenolic and flavonoid content of Cl.Mce were also assessed. The larger the quantity, the more phenolic and flavonoid moieties are present in the Cl.Mce.

Despite the fact that Cichorium intybus is a powerful antioxidant, no study has been conducted to determine how it may impact CS-induced ALI. We investigated Cl.Mce for the first time to see if its antioxidant effects, which have been shown to be beneficial in numerous inflammatory models in vivo, could improve the CS-induced type of ALI. ROS are important secondary mediators that enhance inflammatory responses in the lungs. Aside from the direct impact of hazardous compounds in cigarettes on pulmonary tissues, oxidative stress generated by CS exacerbates inflammatory responses through the following process: i) Injured endothelium, the first line of defense; ii) activated alveolar macrophages increased the amount of binding molecules on the surface; iii) activated macrophages generation of chemokines and pro-inflammatory cytokines promotes neutrophil invasion. ALI is characterized by the integration [62] and adhesion of neutrophils in the airway microcirculation [72] due to ROS-induced neutrophil deformability. Fast and optimal neutrophil and macrophage influxes remove alveolar detritus and hazardous chemicals. CS or oxidative stress reduces their phagocytic capabilities causing lung inflammation and vascular permeation [73]. Several studies have been undertaken to minimize CS-induced neutrophil inflammation. ALI is linked to BALF neutrophil counts. CS exposure substantially enhanced BALF inflammatory cells, macrophages and neutrophils [74–76]. Cl.Mce reduced inflammation, macrophages and neutrophils. Smoking increases neutrophils and white blood cells causing acute lung inflammation. Cl.Mce therapy lowered WBCs and neutrophils and dose-dependently reduced CS-induced inflammation.

Pulmonary edema is connected to protein buildup and alveolar capillary permeability [61,77]. LCW indicates pulmonary edema severity. Observations of pulmonary edema following CS exposure indicated a statistically significant increase [14]. ROS changes the structure of the lungs by damaging key alveolar components, despite Cl.Mce treatment lowering LCW dose-dependently. After mild CS exposure, changes in the architecture of the alveoli, such as stiff membranes, inflammatory cells like macrophages and neutrophils getting into alveolar gaps or vessel walls and alveolar fluid building up were noticed. In lung histology examination and a second lung injury score Cl.Mce pre-treatment reduced inflammation, alveolar wall damage, neutrophil and macrophage invasion and other lung abnormalities. Alveolar edema and protein leakage contribute to ALI-related hypoxemia. 24 h after the last CS exposure, the mice SO2 levels were examined [59]. We found that CS-exposed mice developed hypoxemia (lower SO2) whereas Cl.Mce-treated animals showed greater oxygen saturation and dose-dependently reduced CS-induced hypoxemia.

Antioxidants including glutathione, carotene, and ascorbic acid protect lung tissue against oxidants, toxins and pollutants. Bio-molecules like lipids, proteins and DNA are damaged by CS inhalation owing to an imbalance between oxidants and antioxidants. Numerous studies demonstrate ALI/ARDS is linked to cigarette smoke-induced oxidative damage [78]. This research analyzed signs of oxidative stress coupled with TOS, TAC and MDA to maintain a healthy balance between free radicals and antioxidants. Oxidants in the CS may produce lipid peroxidation resulting in the generation of MDA, which may worsen lung injury. Several studies link lower neutrophil penetration to lower MDA levels [79]. According to the TBARS test, MDA was significantly higher in CS-exposed mice, confirming prior research. Cl.Mce lowers MDA levels which suggests that it can stop lipid peroxidation in CS-induced ALI and prevent problems related to ALI [64]. The combined action of endogenous and exogenous antioxidants in the lungs showed the TAC in BALF as individual antioxidant molecule analyses are impracticable. CS may induce a considerable drop in TAC owing to the absence of antioxidant compounds [80]. Cl.Mce and Dexa substantially elevated TAC showing Cl.Mce has antioxidant capabilities. CS dramatically increased TOS in CS-exposed mice indicating the lungs lack anti-oxidative defenses. Cl.Mce decreases TOS to counteract ROS [81].

Studies suggest that polyphenols capacity to bind reactive oxygen species reduces oxidative damage (ROS). Using enzyme inhibition and metal cation sequestration, phenolic compounds give protection. Plant HPLC reveals that Cl.Mce contains a lot of polyphenols and studies suggest it may help remove free radicals in ALI produced by CS [82]. In vitro testing using DPPH showed that Cl. Mce has strong antioxidant activity [83].

In CS-induced ALI/ARDS models, pro-inflammatory factors (IL-6, IL-1β, and KC) are increased in lungs due to oxidative stress [62, 84]. Normally, IL-1β produced by macrophages stimulates cell differentiation and hence strengthens immunological response; nevertheless, excessive amounts of IL-1β explicitly disrupt pulmonary vascular and epithelial membranes, initiating inflammation. The oxidative stress induced by CS promotes alveolar edema and protein loss, which leads to hypoxemia and exacerbated pulmonary
edema \cite{8,13,14}. As predicted, CS exposure enhanced macrophage incursion in BALF and increased levels of pro-inflammatory cytokines and chemokine, whilst Ci.Mce considerably decreased unrestrained upregulation of pro-inflammatory mediators and altered inadequate upsurge of macrophages. After in vivo results, the in vitro influence of Ci.Mce on RAW 264.7 macrophages was assessed using CSE (4%). The results of ELISA showed that macrophages stimulated by CSE produced increased IL-6, IL-1\(\beta\), and KC as supported by previous studies \cite{85–87}, which were lowered by pre-treatment with Ci.Mce, demonstrating anti-inflammatory effect of Ci.Mce at both in vivo and in vitro levels.

Furthermore, the effects of Ci.Mce on CS-induced NF-\(\kappa\)B activation were explored in vivo and in vitro to investigate probable mechanism by which Ci.Mce decreases oxidative stress and mitigates production of pro-inflammatory regulators. The findings demonstrate that CS treatment considerably reinforced NF-\(\kappa\)B-p65 phosphorylation in pulmonary tissue and RAW 264.7 macrophages, as predicted and in accord with prior investigations \cite{88–90}. Ci.Mce prevented the activation of the NF-\(\kappa\)B-p65 pathway, which collectively markedly reduced the uncontrolled inflammatory reaction and overwhelming oxidative stress in ALI.

The interrelation between NF-\(\kappa\)B signaling pathways and inflammatory mediators such as IL-6 and IL-1\(\beta\) is of notable importance in the regulation of inflammation \cite{91}. NF-\(\kappa\)B, which constitutes a group of transcription factors, assumes a central role in immune responses and inflammation. The activation of NF-\(\kappa\)B triggers the production of IL-6 and IL-1\(\beta\), both recognized as potent pro-inflammatory cytokines. This interaction creates a positive feedback loop: NF-\(\kappa\)B activation stimulates the cytokines, which in turn prompt NF-\(\kappa\)B activity, thereby intensifying the inflammatory response. This reciprocal association underscores potential targets for therapeutic strategies aimed at managing conditions characterized by excessive inflammation. Flavonoids and phenols, found in various plant-based foods, exhibit strong anti-inflammatory effects by targeting key components of the inflammatory response. As we found that phenols and flavonoids are present in \textit{Cichorium intybus} which inhibit the production of pro-inflammatory cytokines like IL-6 and IL-1\(\beta\), as well as chemokines like KC. These effects are largely mediated through their ability to modulate the activity of NF-\(\kappa\)B, a central regulator of inflammation. By blocking NF-\(\kappa\)B activation and interfering with upstream signaling pathways, these compounds mitigate the inflammatory cascade. Consequently, flavonoids and phenols present \textit{Cichorium intybus} could hold promise in modulating these pathways to control inflammatory responses like CS-induced ALI.

5. Conclusion

Taken together, \textit{Cichorium intybus} has a considerable ability to reduce CS-induced ALI by lowering alveolar edema, serum white blood cell count, alveolar infiltration of neutrophil and macrophage, production of MPO, MDA and TOS, alveolar secretion of chemokines (KC) and proinflammatory cytokines (IL-6, IL-1\(\beta\)), and improving histopathological changes and level of TAC. We are the first to report that \textit{Cichorium intybus} can be used as a potential therapeutic agent to treat ALI/ARDS because the anti-oxidant and the anti-inflammatory response of \textit{Cichorium intybus} has drastically lowered CS-induced acute lung injury via possibly blocking NF-\(\kappa\)B pathway at both in vivo and in vitro level.

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Appendix A. Supplementary data

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