Evidence-Based Complementary and Alternative Medicine

Pharmacological Activities of Natural Products in Respiratory Disorders

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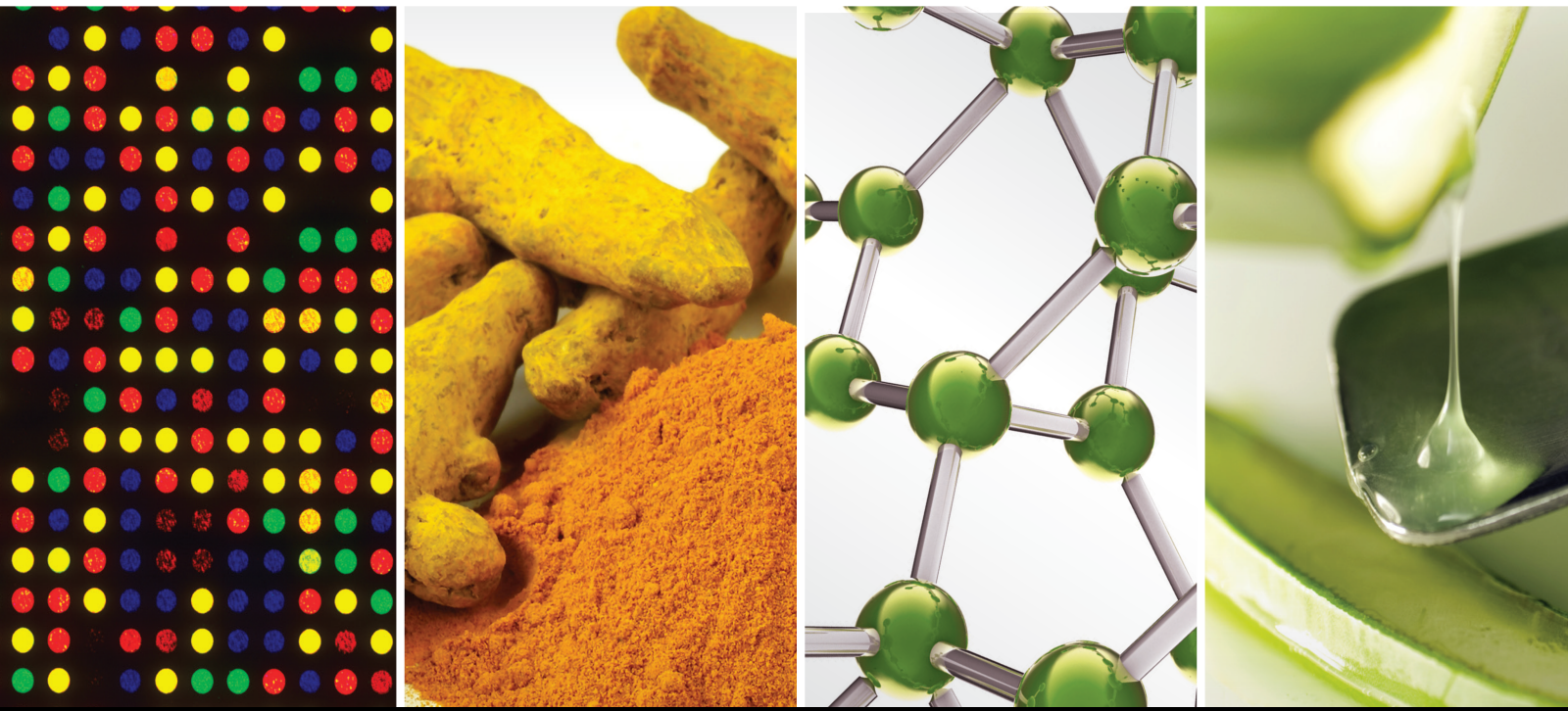

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Research Article

The Effect of *Curcuma longa* on Inflammatory Mediators and Immunological, Oxidant, and Antioxidant Biomarkers in Asthmatic Rats

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The effects of *Curcuma longa* (*C. longa*) on total and differential WBC, inflammatory and immunologic mediators, and oxidant and antioxidant biomarkers in bronchoalveolar lavage fluid (BALF) of rats model of asthma were assessed. Animals were divided to 5 groups including control (C), asthma (sensitized to ovalbumin), and asthmatic groups treated with 0.75, 1.50, and 3.00 mg/ml *C. longa* (CL) and 1.25 μg/ml dexamethasone (D) (8 rats in each group). Total and differential WBC count, concentrations of phospholipase A_2 (PLA_2), total protein (TP), interferon-gamma (IFN-γ), interleukin-4 (IL-4), immunoglobulin E (IgE), NO_2, NO_3, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and thiol in BALF were assessed. Total and most differential WBC counts and BALF levels of PLA_2, TP, IgE, IL-4, and oxidants in asthma group were higher but antioxidants and IFN-γ levels as well as IFN-γ/IL-4 ratio were lower than control group ($p < 0.001$ for all cases). Total WBC and levels of PLA_2, IgE, NO_2, and NO_3 were significantly reduced following treatment with *C. longa*, compared to asthma group ($p < 0.001$ for all cases). In groups treated with dexamethasone and two higher concentrations of *C. longa*, neutrophil and eosinophil counts as well as TP, IL-4, and MDA levels were significantly decreased but IFN-γ, IFN-γ/IL-4 ratio, and antioxidants were increased (except IFN-γ/IL-4 ratio), compared to asthma group ($p < 0.05$ to $p < 0.001$). Compared to dexamethasone, *C. longa* exerted more pronounced effects on lung inflammation, oxidative stress, and immune system in asthmatic rats.

1. Introduction

Curcuma longa L. *(C. longa)* is a perennial herb of the Zingiberaceae family (ginger), with the common name Turmeric. The plant is used as a traditional cure in India, China, and other Southeast Asian nations for the treatment of asthma and common cold [1]. In Ayurvedic medicine, turmeric is a well-known remedy for several respiratory problems such as asthma and allergy, runny nose, cough, and sinusitis [2]. Moreover, it is used not only as a spice in food preparation as the major ingredients of curry and mustard but also as a functional food due to its potential health benefits [3].

C. longa contains various constituents including terpenes, phenolic compounds, steroids, fatty acids, and other compounds. The level of curcumin, the main curcuminoid of *C. longa*, varies between varieties and cultivation locations and conditions (1.28 to 6.6%) and North Indian plains (0.61 to 1.45%), on a dry weight basis [4].

C. longa contains carbohydrates, moisture, protein, fat, and minerals. The essential oil of rhizomes the plant using steam distillation contains sesquiterpenes, zingiberene,

a-phellandrene, cineol, sabinene, and borneol. It includes curcumin I (94%), curcumin II (6%), and curcumin III (0.3%), responsible for the vibrant yellow color of turmeric [5].

Various anti-inflammatory [6], antiasthmatic [7], relaxant [8, 9], and antioxidant [10] effects were described for this plant. The effect of turmeric on ovalbumin (OVA)-sensitized mice was demonstrated to be antioxidant [11] and had immunomodulatory effects by altering the balance of $CD4þ^{+}$ $CD25^{+}$ regulatory T cells (Tregs)/T-helper (Th), [12]. Treatment with methanolic extract of *C. longa* (200 mg/kg, for 14 days) stimulated innate and adaptive immunity in mice. The effect of the extract on adaptive immunity was evaluated by immunizing and challenging the mice with sheep red blood cells (sRBCs) on days 7 and 14, respectively. *C. longa* enhanced the adaptive immunity by increasing leukocyte number, antibody titer, spleen index, and delayed-type hypersensitivity response [13]. In another study, *C. longa* root aqueous extract standardized was indicated to stimulate NO production in RAW264.7 macrophages [14].

Asthma is a chronic inflammatory airway disorder involving inflammatory cells such as eosinophils, mast cells, T-lymphocytes, epithelial cells macrophages, and neutrophils [15]. These inflammatory cells produce inflammatory mediators, which play a key role in pulmonary inflammation and asthma [16]. The first line of defense against inhaled environmental oxidants, and endogenous oxidants, is the high concentrations of antioxidants in the epithelial lining fluid in the lung [17]. Alterations in the antioxidant/oxidant balance in bronchoalveolar lavage fluid (BALF) in asthma can indicate a continuous inflammation procedure. There are reports regarding the imbalance in Th1/Th2 ratio toward increased Th2 activity, in asthma [18]. Th2 cells secrete IL-4, IL-5, and IL-13, which lead to allergic inflammatory diseases. Th1 produces IL-2 and IFN-γ which in turn inhibit Th2 activities [19]. Therefore, increasing IFN-γ/IL-4 or Th1/Th2 ratios could be considered a treatment approach for asthma.

Although there are several studies on the immunological and antioxidant effects of *C. longa*, the effect of the plant on immunological changes and oxidative stress in asthma was not fully addressed yet. Therefore, in the present study, the potential anti-inflammatory, antioxidant, and immunomodulatory effects of the extract of *C. longa* hydroethanolic on sensitized rats were evaluated by measuring total and differential WBC count, PLA_2, TP, IgE, IFN-γ, IL-4, and oxidant and antioxidant biomarker levels in BALF.

2. Materials and Methods

2.1. Asthma Induction in Rats. Male Wistar rats (age 8-9 weeks and weight 200–250 g) model of asthma was induced by three intraperitoneal (i.p.) injections of 1 mg/kg chicken egg albumin (Ovalbumin = OVA) in 0.9% sterile saline + 100 mg $Al(OH)_3$, on days 1, 2, and 3. Animals were then exposed to aerosolize 1% OVA for 20 min/day, on days 6, 9, 12, 15, 18, and 21 as previously described [20].

Animals were obtained from Animal House, School of Medicine, Mashhad University of Medical Sciences, Mashhad (MUMS), Iran, and kept in a temperature-controlled room ($22 \pm 2C^{\circ}$) with 12 h light/12 h dark cycles and had free access to food and tap water. Ethics Committee of MUMS approved the study (ethics allowance no. 921249), and the regulations of the Institute of Laboratory Animals Resources Commission on Life Sciences were followed in animal handling.

2.2. Plant Materials and Extraction. C. longa rhizomes (100 grams) were purchased from a local herbal market in Mashhad, Khorasan Razavi province, Iran. Then, it was ground to powder and extracted with ethanol (96%). The resultant extract was concentrated under low pressure in an Eyela (Heidolph, Schwabach, Germany) rotary evaporator. The extraction yield was 14% [21]. The qualitative and quantitative characteristics of curcumin in the extract [8] was determined as previously described (Figure 1).

2.3. Experimental Animal Groups. Animals were randomly divided into the following groups ($n = 8$ in each group):

(A) Control or saline-treated rats (group C)

(B) Asthmatic rats (OVA sensitized, group A)

(C) Asthmatic animals treated with different concentrations (0.75, 1.5, and 3 mg/ml) of *C. longa* (groups CL 0.75, CL 1.50, and CL 3.00, respectively)

(D) Asthmatic animals treated with dexamethasone (1.25 μg/ml) (group A + D)

In treated animals, the extract of *C. longa* and dexamethasone were added to the drinking water during sensitization period. On average, every rat consumed 40 ml of drinking water each day, and no significant difference was made between different groups in water intake.

2.4. Evaluation of WBC Count (Total and Differential), Inflammatory Mediators, and Cytokines Levels in the BALF. At the end of the sensitization period, rats were deeply anesthetized by i.p. injection of 50 mg/kg ketamine. The animals were sacrificed by a competent researcher with minimum pain, suffering, and distress. The method was performed according to Annex IV of the guidelines from Directive EU/2010/63 of the European Parliament guideline. The trachea and lungs were then removed after the chest was opened. The right lung was lavaged five times with 1 ml saline (a total of 5 ml).

Total WBC was counted in 1 ml BALF after staining with Turk solution in duplicate using a hemocytometer as previously described [22]. Under light microscopy, differential WBC was determined using morphological criteria by counting 100 cells and calculating the percentage of each cell type in a smear of the cells stained with Wright-Giemsa [23].

The remaining BALF was centrifuged at $2500\times g$ at $4^{\circ}C$ for 10 min, supernatant was removed, and inflammatory mediator (PLA_2), oxidant and antioxidant biomarkers, cytokine, and immunological (IFN-γ, IL-4, and IgE) levels were measured according to the method described previously [20].

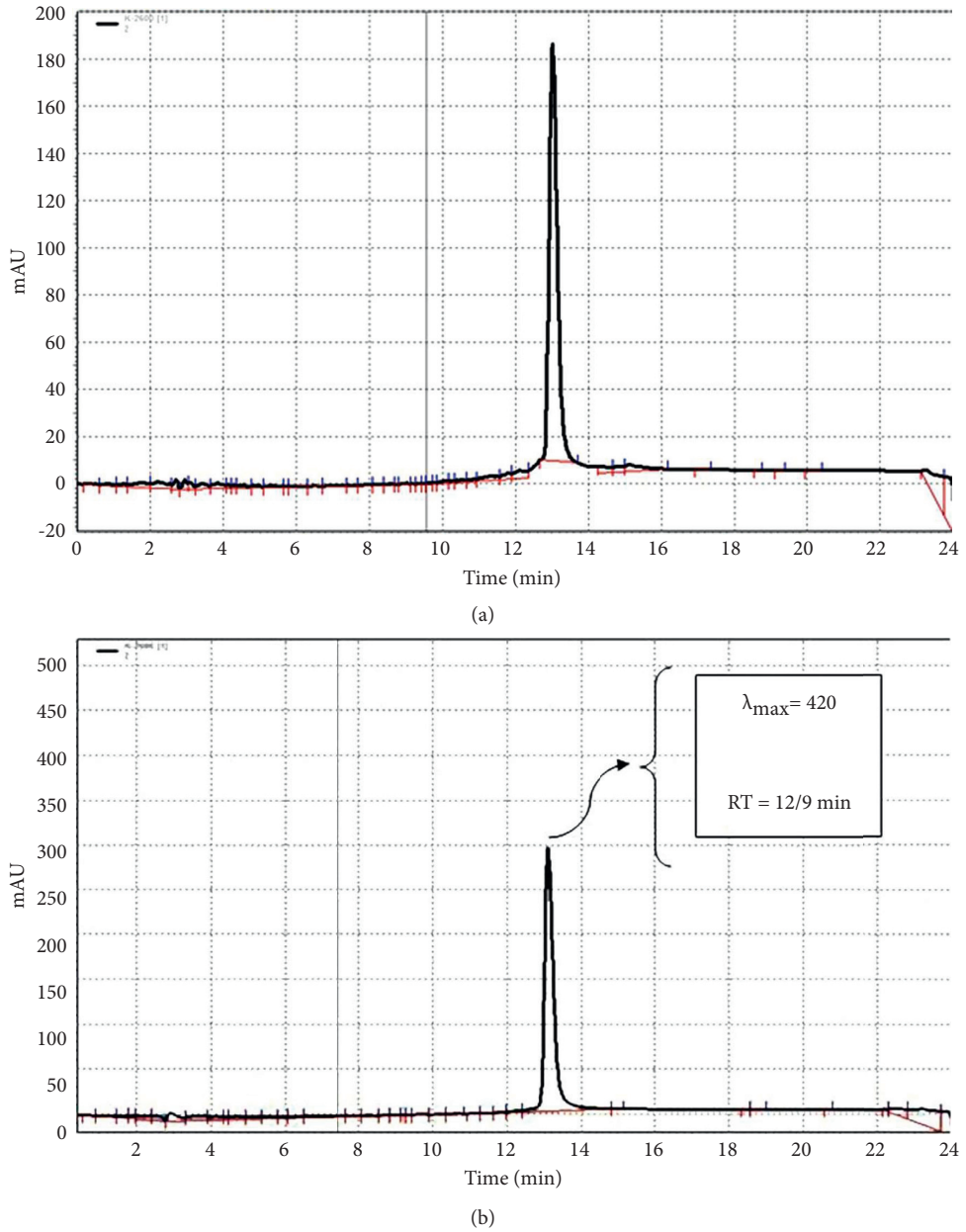

Figure 1: RP-HPLC of (a) the extract of *C. longa* and (b) curcumin (8).

2.5. Evaluation of Oxidative Stress Biomarkers in BALF. The products of NO metabolism (NO_{2-}/NO_{3-}) [24], malondialdehyde (MDA) levels [25], superoxide-dismutase (SOD) [21], and catalase (CAT) [23] activities, and total thiol concentration [20] in the BALF supernatant were measured using previously described methods.

2.6. Statistical Analysis. One-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test was used for comparisons among and within groups using InStat (GraphPad Software, USA). The data are shown as mean-$\pm$SEM. $p < 0.05$ was selected as statistical significance criterion.

3. Results and Discussion

3.1. The Changes in the Asthmatic Group. In asthmatic animals, total WBC count, percentages of eosinophil and neutrophil, and BALF levels of PLA_2, TP, IgE, IL-4, NO_2, NO_3, and MDA were significantly higher but lymphocyte percentage, IFN-γ level, and IFN-γ/IL-4 ratio as well as SOD, CAT, and thiol levels were lower than those in nonasthmatic (i.e., group C) rats ($p < 0.001$ for all cases; Figures 2–7).

3.2. The Extract of C. longa Effects. Treatment of asthmatic animals with all concentrations of *C. longa* significantly reduced total WBC and BALF levels of PLA_2, IgE, NO_2, and

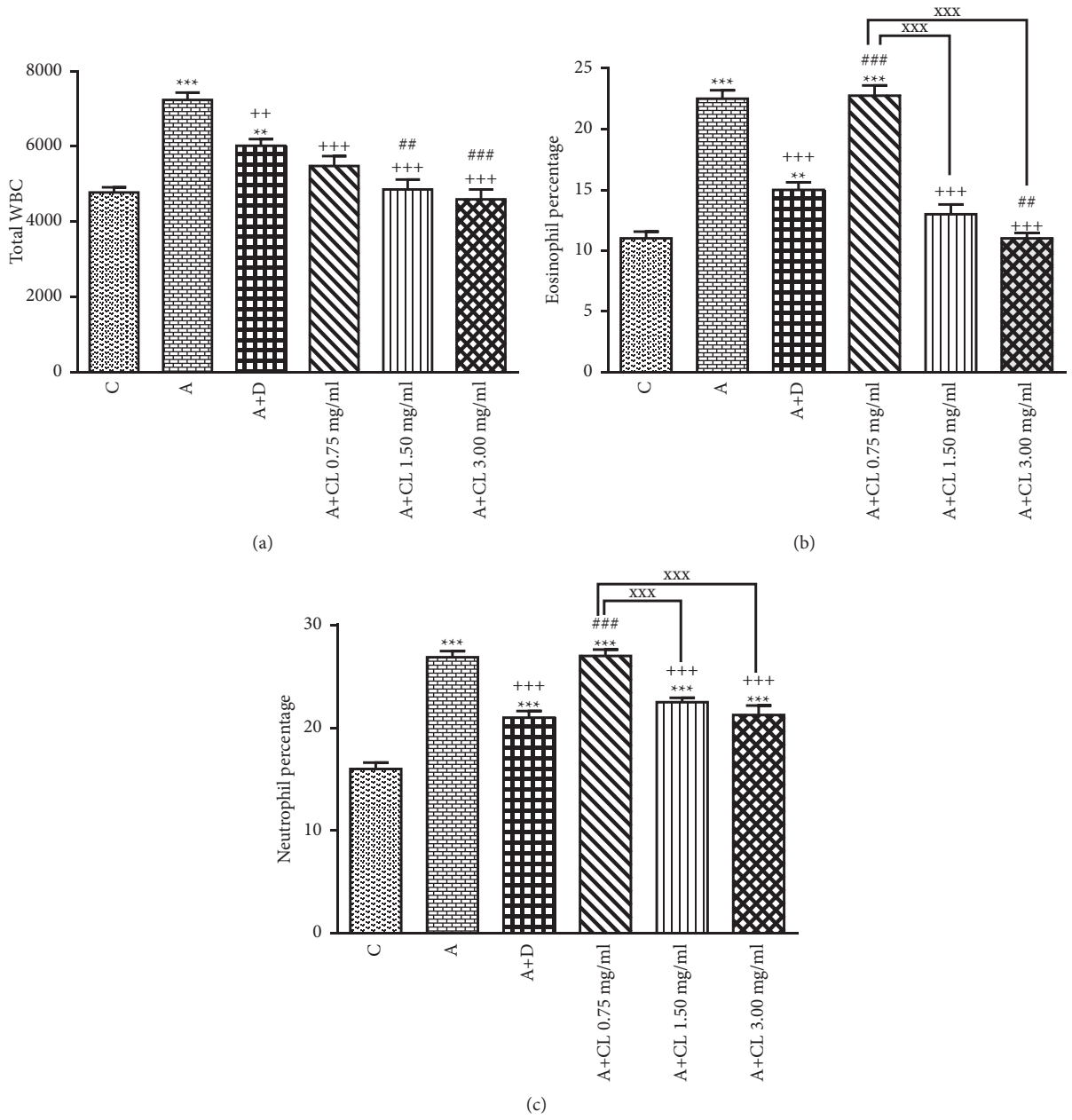

Figure 2: The effect of *C. longa* on total WBC number (count/ml of BALF) (a) and percentage of eosinophil (b) and neutrophil (c) in control animals (C), asthma group (A), and asthmatic groups treated with dexamethasone (D) and *C. longa* (CL) (0.75 mg/ml, 1.50 mg/ml, and 3.00 mg/ml) (for each group, $n = 8$). Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to group C. $^{++}p < 0.01$, $^{+++}p < 0.001$, compared to group A. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to group D. $^{xxx}p < 0.001$, comparison among three concentrations of *C. longa*. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer's post-test.

NO_3 compared to asthmatic group ($p < 0.001$ for all cases). Two higher concentrations of *C. longa* also significantly decreased neutrophil and eosinophil counts, as well as TP, IL-4, and MDA levels, but increased lymphocyte count, IFN-γ/IL-4 ratio, and BALF levels of IFN-γ, SOD, CAT, and thiol compared to untreated asthmatic group ($p < 0.05$ to $p < 0.001$; Figures 2–7).

The percentages of eosinophil and monocyte in group treated with low concentration of *C. longa*, lymphocyte percentage, and levels of PLA_2, TP, IgE, NO_2, and NO_3 in groups treated with two lower concentrations of *C. longa*, as well as neutrophil percentage, IFN-γ/IL-4 ratio, and levels of IFN-γ, IL-4, MDA, SOD, CAT, and thiol in groups treated with all concentrations of *C. longa* were significantly different compared to control group ($p < 0.01$ to $p < 0.001$; Figures 2–7).

3.3. Dexamethasone Effects. Total WBC, percentages of eosinophil and neutrophil, and levels of PLA_2, TP, IgE, IFN-γ, IL-4, NO_2, NO_3, and MDA were decreased but lymphocyte percentage and SOD, CAT, and thiol levels

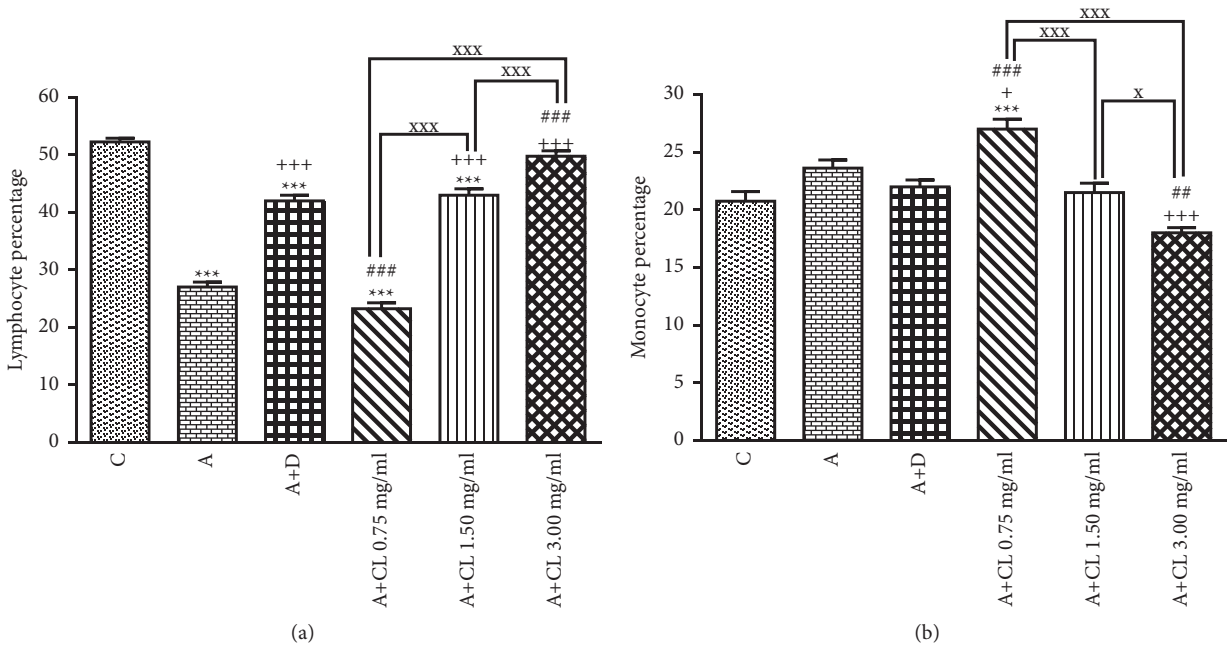

Figure 3: The effect of *C. longa* on percentage of lymphocyte (a) and monocyte (b) in control animals (C), asthma group (A), and asthmatic groups treated with dexamethasone (D) and *C. longa* (CL) (0.75 mg/ml, 1.50 mg/ml, and 3.00 mg/ml) (for each group, $n = 8$). Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, compared to group C. $^{+}p < 0.05$, $^{+++}p < 0.001$, compared to group A. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to group D. $^{x}p < 0.05$, $^{xxx}p < 0.001$, comparison among three concentrations of *C. longa*. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer's post-test.

were increased in dexamethasone-treated group compared to untreated asthmatic group ($p < 0.05$ to $p < 0.001$). However, dexamethasone did not affect IFN-γ/IL-4 ratio and monocyte percentage compared to asthmatic group (Figures 2–7).

In asthmatic group treated with dexamethasone, however, total WBC, percentages of eosinophil, neutrophil, and lymphocyte, and levels of TP, IgE, IFN-γ, IL-4, SOD, CAT, and thiol were significantly different compared to control group ($p < 0.01$ to $p < 0.001$; Figures 2–7).

3.4. Differences among the Effects of Various Concentrations of C. longa Extract. Eosinophil, neutrophil, lymphocyte, and monocyte percentages, IFN-γ/IL-4 ratio, and levels of PLA_2, TP, IgE, IL-4, IFN-γ NO_2, NO_3, MDA, SOD, CAT, and thiol were significantly higher in asthma groups treated with two higher concentrations of the extract (1.50 and 3.00 mg/ml) compared to its low concentration ($p < 0.05$ to $p < 0.001$). The effects of high extract concentration on lymphocyte and monocyte percentages and levels of PLA_2, TP, IgE, IL-4, IFN-γ/IL-4, NO_2, NO_3, MDA, SOD, CAT, and thiol were also significantly higher than the medium concentrations ($p < 0.05$ to $p < 0.001$).

3.5. Comparison of the Effect of C. longa Extract with Dexamethasone on Asthma. Treatment with all concentrations of the extract on IFN-γ level, treatment with its two higher concentrations on total WBC, IL-4 level, and IFN-γ/IL-4 ratio, and treatment with its high concentration on eosinophils, lymphocytes, and monocytes percentages and levels of TP, IgE, and thiol had significantly more marked effects compared to dexamethasone ($p < 0.01$ to $p < 0.001$; Figures 2–7). The effect of all concentrations of the extract on MDA, the effect of its two lower concentrations on PLA_2, NO_2, NO_3, CAT, and thiol, and the effect of its low concentration on eosinophil, neutrophil, monocyte, and lymphocyte percentages and levels of TP, IgE, IL-4, and SOD were significantly lower than that of dexamethasone ($p < 0.05$ to $p < 0.001$; Figures 2–7).

4. Discussion

After OVA sensitization, a rat model of asthma was produced; in asthmatic animals, total WBC, percentages of eosinophil and neutrophil, and BALF levels of PLA_2, TP, IgE, IL-4, NO_2, NO_3, and MDA were increased while lymphocyte percentage, IFN-γ/IL-4 ratio, and IFN-γ, SOD, CAT, and thiol levels were decreased compared to control animals. These results demonstrated asthma induction in rats.

Previously, increased total WBC and neutrophil and eosinophil percentage in lung lavage of OVA-sensitized rats were shown [23, 26]. Increased percentages of eosinophils were also demonstrated in sensitized animals and asthmatic patients that correlated with the severity of asthma and supported the results of the present study [27]. Activated eosinophils are critical in allergic reactions and induce respiratory hyperactivity in asthma [28].

In etiology of asthma and COPD, lymphocytes play a crucial role. A greater number of $CD8^{+}$ type-1

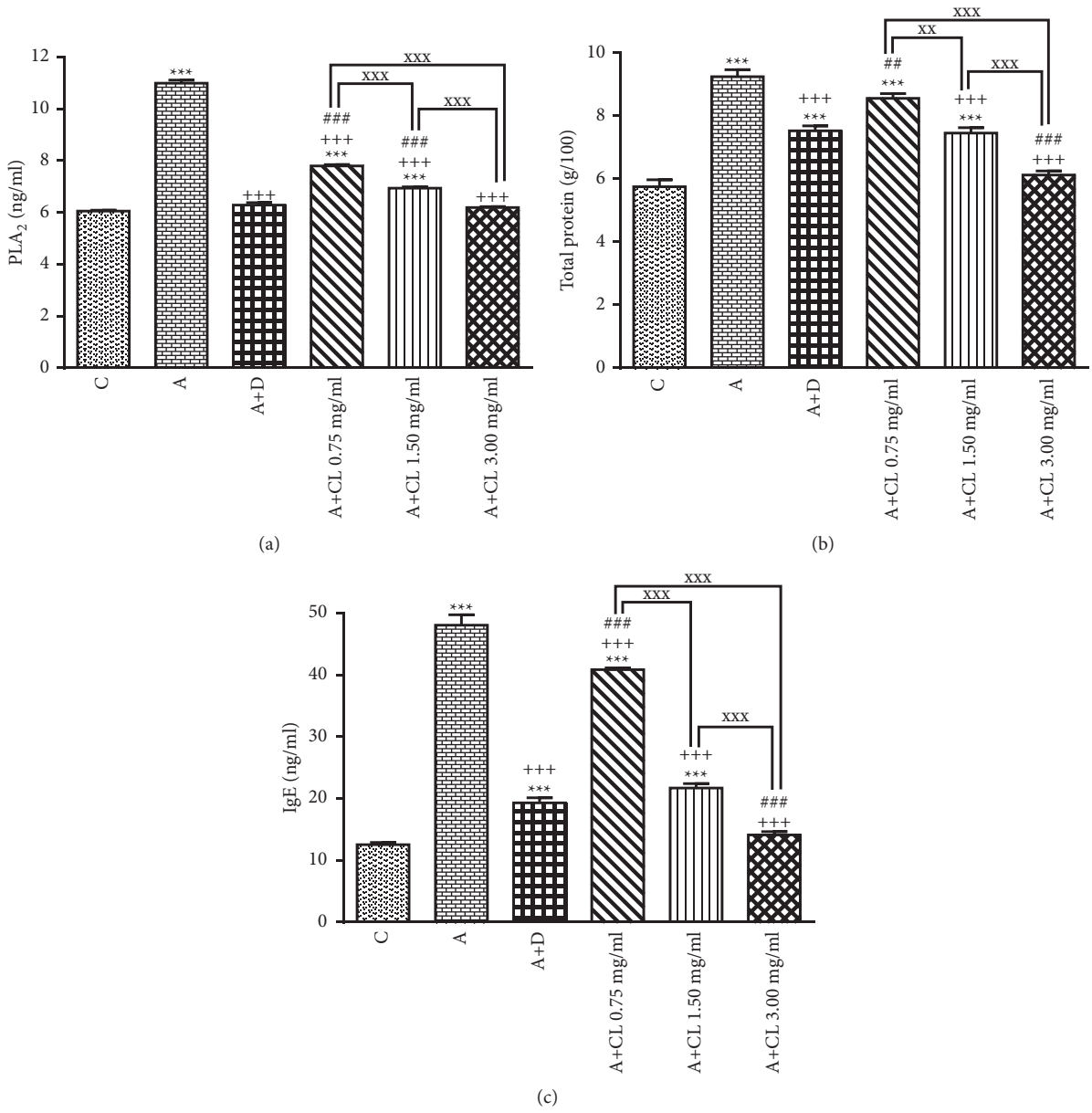

Figure 4: The effect of *C. longa* on TP (a), PLA_2 (b), and IgE (c) in control animals (C), asthma group (A), and asthmatic groups treated with dexamethasone (D) and *C. longa* (CL) (0.75 mg/ml, 1.50 mg/ml, and 3.00 mg/ml) (for each group, $n = 8$). Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to group C. $^{++}p < 0.01$, $^{+++}p < 0.001$, compared to group A. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to group D. $^{xx}p < 0.01$, $^{xxx}p < 0.001$, comparison among three concentrations of *C. longa*. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer's post-test.

T-lymphocytes and lung macrophages indicate chronic inflammation of airways [29]. The present investigation found that the lymphocyte percentage of asthmatic animals in BALF has decreased. Moreover, in a previous study which used a similar sensitization protocol to that of the current study, increased absolute lymphocyte count was observed. Therefore, reduction of lymphocyte percentage could be due to increased total WBC of asthmatic animals [27]. If the absolute lymphocyte count was considered, it would be higher in the asthmatic rats compared to control group.

There has been an increase in the amount of neutrophils in lung lavage of asthma patients and a significant link between inflammation of the airways and severity of asthma [30, 31]. Neutrophils include significant cytokine sources including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-8. Activated neutrophils are able to release human neutrophil elastase (HNE) and myeloperoxidase (MPO) that exacerbate and prolong asthma symptoms and contribute to the airway inflammation [32].

Alterations in monocyte numbers in the peripheral blood are associated with asthma attacks induced by allergic reactions. Activation of monocytes also contributes to asthma pathogenesis by releasing reactive nitrogen species,

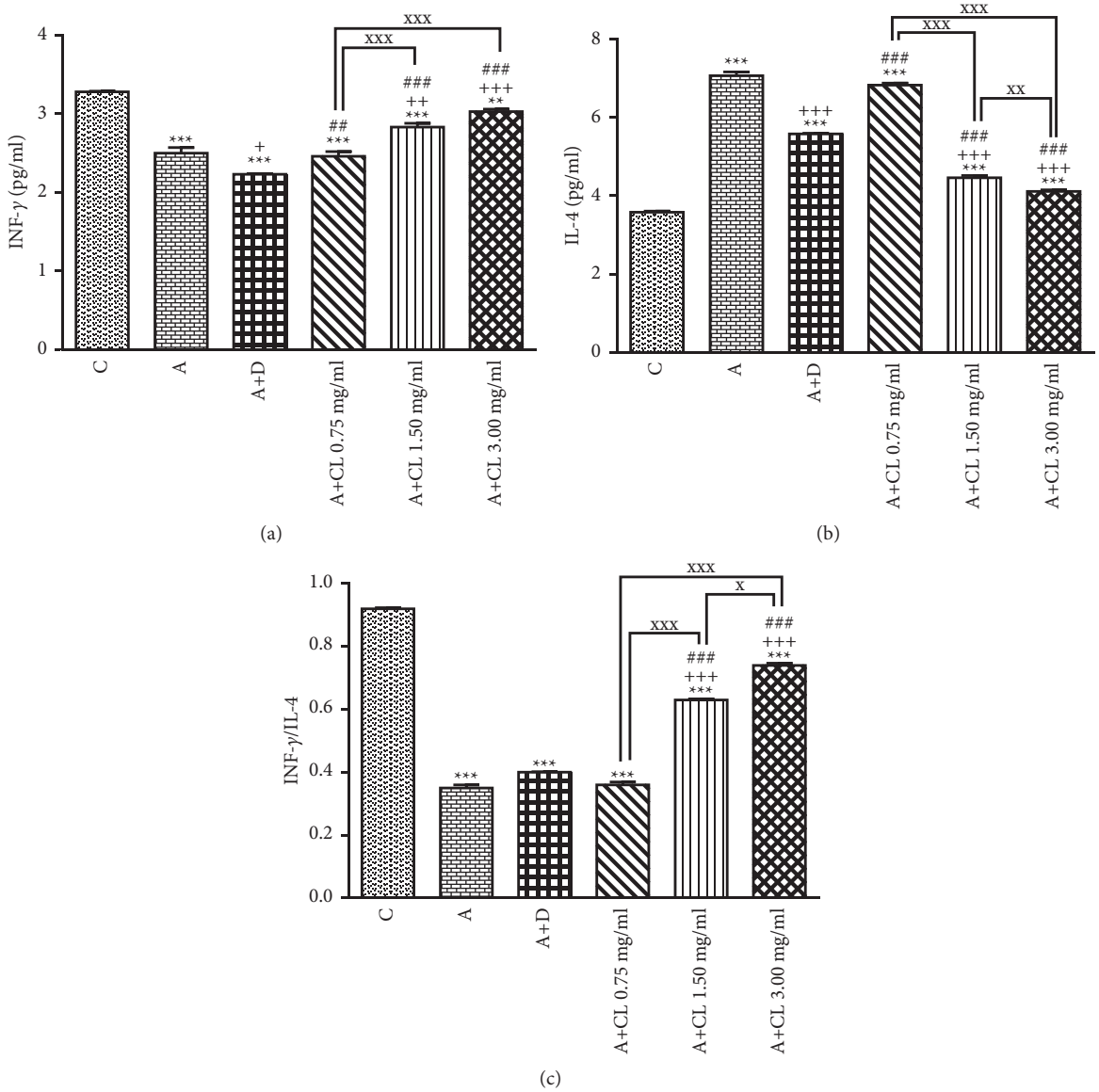

Figure 5: The effect of *C. longa* on IFN-γ (a), IL-4 (b), and IFN-γ/IL-4 ratio (c) in control animals (C), asthma group (A), and asthmatic groups treated with dexamethasone (D) and *C. longa* (CL) (0.75 mg/ml, 1.50 mg/ml, and 3.00 mg/ml) (for each group, $n = 8$). Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to group C. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, compared to group A. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to group D. $^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$, comparison among three concentrations of *C. longa*. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer's post-test.

total free radicals, and proliferation of Th2-like lymphocytes in patients with asthma [33].

Both animal and human studies have shown increased PLA_2 and TP in asthma [27, 34]. Consequently, higher levels of PLA_2 and TP in the BALF of asthmatic rats also confirm the induction of an animal model of asthma in the present study. PLA_2 has a significant function in asthma pathogens through the development of eicosanoid formation, dendritic cells maturation and migration, T cells proliferation, and production of cytokines and chemokines by monocytes, macrophages, neutrophils, and eosinophils [35].

The findings from the current investigation were comparable to those in earlier studies that induced asthma using a similar sensitization method [36, 37] which also confirmed animal sensitization with changes in IL-4, IFN-γ, and IFN-γ/IL-4 ratio. *In vivo* and *in vitro* studies have also shown increased IL-4 but reduced IFN-γ and IFN-γ/IL-4 in OVA-sensitized guinea pigs [38]. Increased release of Th2 cytokines mainly IL-4, IL-5, and IL-13 and decreased release of IFN-γ as a Th1 cytokine also contribute in the onset and progression of asthma [29].

In accordance with the results of this study on indicators of oxidant/antioxidants, there is clear evidence of the function of oxidative stress in inflammation of airways in asthmatic conditions [39]. Increasing production of oxidants in asthmatic groups in comparison to healthy controls

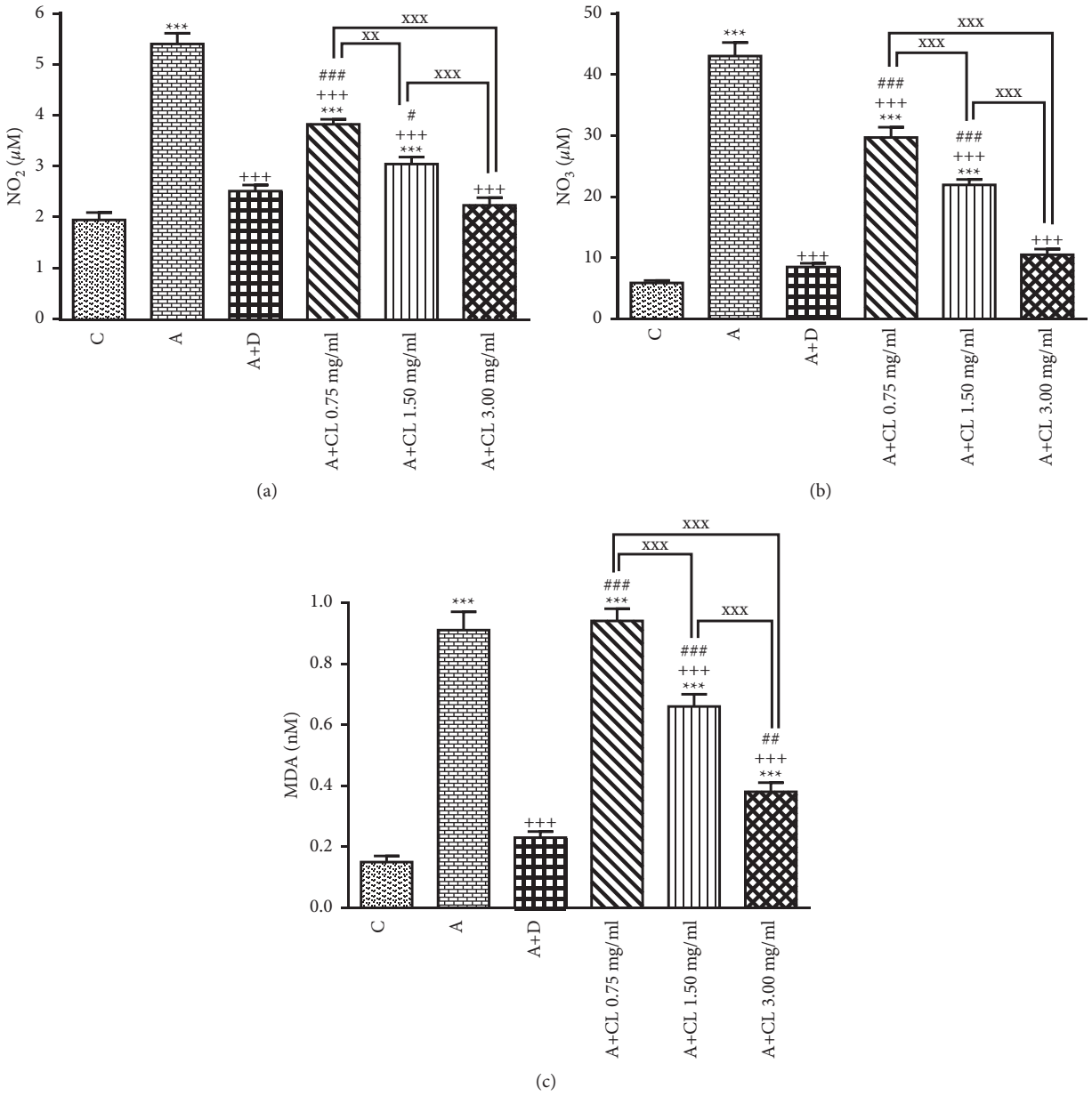

Figure 6: The effect of *C. longa* on NO_2 (a), NO_3 (b), and MDA (c) concentration in control animals (C), asthma group (A), and asthmatic groups treated with dexamethasone (D) and *C. longa* (CL) (0.75 mg/ml, 1.50 mg/ml, and 3.00 mg/ml) (for each group, $n = 8$). Data are mean ± SEM. $^{***}p < 0.001$, compared to group C. $^{+++}p < 0.001$, compared to group A. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to group D. $^{xx}p < 0.01$, $^{xxx}p < 0.001$, comparison among three concentrations of *C. longa*. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer's post-test.

was reported in both animal and human investigations [40]. Increased NO_2 and NO_3 levels in exhaled breath condensate of asthmatic patients were also associated with oxidative stress [41]; also an increased plasma MDA and amplified protein carbonyl levels were also identified in BALF and peripheral blood sample [42]. Alterations in SOD activity caused apoptosis and damaged bronchial epithelial cells, which make a considerable contribution to rehabilitation in the airways and hyper responsiveness of asthma [43]. Decreases in CAT activity possibly augment oxidative stress in the airways in asthma and perpetuate the inflammatory processes [44]. Thiol is an initial sign of oxidative stress in asthma and COPD [45]. Reduction of SOD levels [46], CAT activity, and other antioxidants, such as ascorbate, a-tocopherol, and glutathione, were shown in asthmatic patients [47], which supports the current study's findings.

Total WBC, neutrophil and eosinophil percentages, and PLA_2, TP, IL-4, IgE, NO_2, NO_3, and MDA levels were significantly reduced but IFN-γ/IL-4 ratio, IFN-γ, lymphocyte, SOD, thiol, and CAT levels were increased in asthmatic rats treated with the extract of *C. longa* compared to untreated asthmatic group.

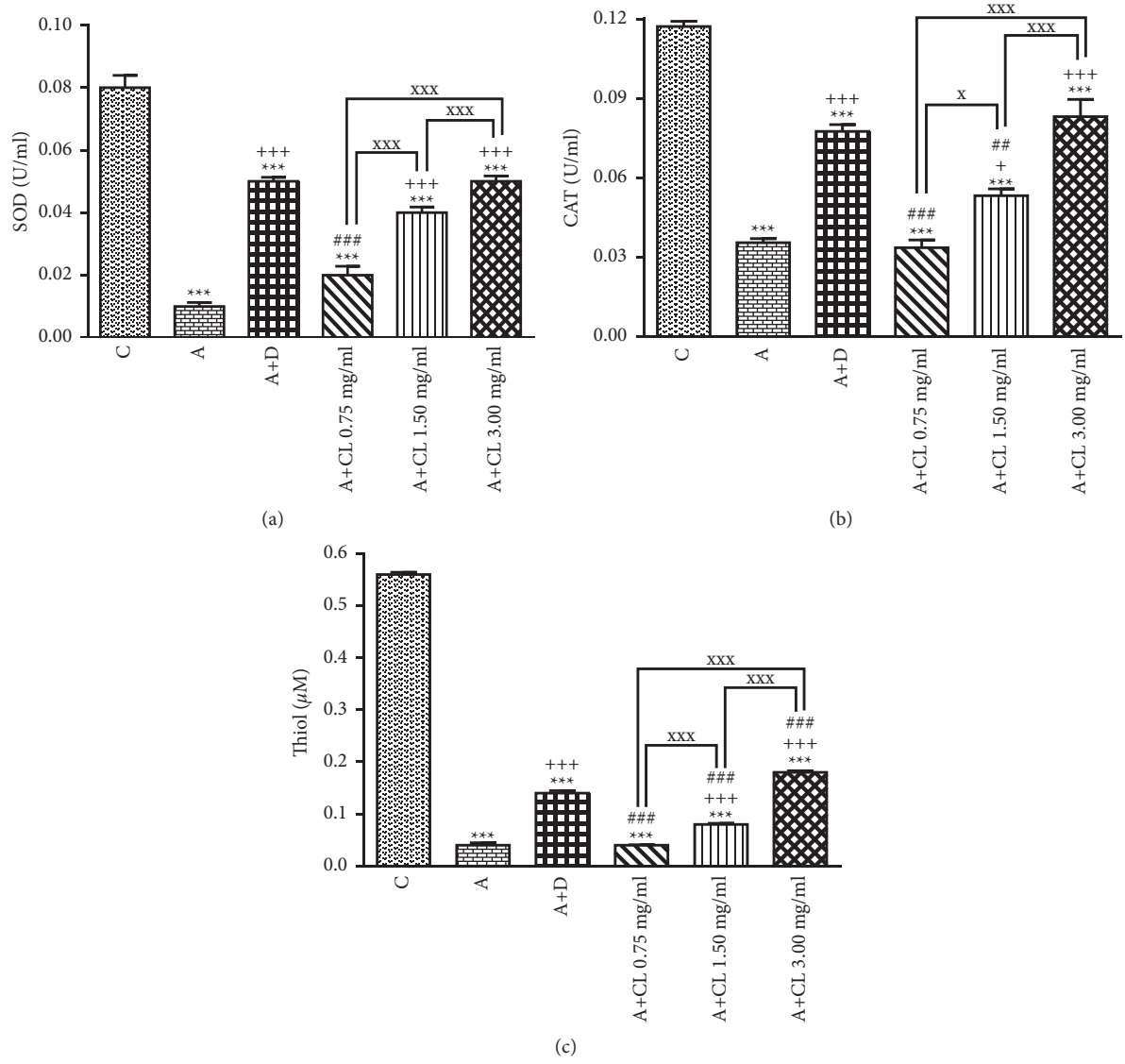

Figure 7: The effect of *C. longa* on SOD (a), CAT (b), and thiol (c) concentration in control animals (C), asthma group (A), and asthmatic groups treated with dexamethasone (D) and *C. longa* (CL) (0.75 mg/ml, 1.50 mg/ml, and 3.00 mg/ml) (for each group, $n = 8$). Data are mean ± SEM. $^{***}p < 0.001$, compared to group C. $^{+}p < 0.05$, $^{+++}p < 0.001$, compared to group A. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to group D. $^{x}p < 0.05$, $^{xxx}p < 0.001$, comparison among three concentrations of *C. longa*. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer's post-test.

The preventive effects of *C. longa* on lung inflammatory cells, inflammatory mediators, and immunological and oxidant-antioxidant markers in asthmatic animals can be identified from these studies.

The effect of *C. longa* on several aspects of asthma was shown previously. Reduction of total and most differential WBC counts in BALF of sensitized rats treated with methanolic extract of *C. longa* [7] decreased PLA_2 activity in BALF of OVA-sensitized mice by intranasal curcumin [48], and reduction of total protein in lung tissue homogenate of OVA-sensitized rats by *C. longa* extract [7] was documented in this regard. Curcumin has also considerably reduced BALF level of TP in a rat model of lipopolysaccharide (LPS)-induced experimental acute lung injury [49]. Moreover, treatment with curcumin suppressed the elevated level of IgE in BALF of OVA-challenged mice [50]. Moreover, asthma and allergic conjunctivitis have been improved by turmeric and curcumin therapy through regulation of Th2, IgE, and immune response to mast cells [51, 52].

Curcumin downregulated IL-4 and IL-5 levels but upregulated IFN-γ in the BALF of a murine model of asthma [50] and attenuated allergic airway inflammation by modifying the balance of $CD4\beta^{+}$ $CD25^{+}$ regulatory T cells (Tregs)/T-helper (Th) in ovalbumin (OVA)-sensitized mice [53] dose-dependently. Another study showed that *C. longa* diminished OVA-induced food allergy by maintaining Th1/Th2 balance in mice [12], which supports the preventive impact of this plant on Th1/Th2 asthma imbalance as seen in this study.

Treatment with methanolic extract of *C. longa* normalized the elevated levels of nitrate and MDA in OVA-sensitized rats [7]. Curcumin and *C. longa* enhanced the activity of SOD [54] and increased CAT activity in the kidney of rats exposed to acetaminophen [55], and curcumin increased thiol concentration in a model of lung carcinogenesis induced by benzo(a)pyrene in mice [56] which was also shown. The current study's findings are supported by the previously described studies, which show that *C. longa* has a preventive therapeutic effect on asthma.

Prevention of lung inflammation and airway responsiveness by inhaled PLA_2 inhibitor in asthma was shown previously [57]. It was also reported that inhibiting the effects of IgE is a novel strategy for blocking or ameliorating symptoms of asthma and allergy [58]. These two studies also support the preventive effect of the plant on PLA_2 and IgE in asthmatic rats and suggest its preventive effect on asthma.

In a previous study, we demonstrated the effect of hydroethanolic extract of *C. longa* rhizome and curcumin on total and differential WBC counts, as well as serum oxidant/antioxidant biomarkers in an asthmatic rat model, which reflect the effect of the plant and its constituent curcumin, on systemic inflammation and oxidative stress [21]. However, in the current study, total and differential WBC count and BALF concentrations of PLA_2, TP, cytokines (IFN-γ and IL-4), IgE, oxidant/antioxidant biomarkers (NO_2, NO_3, MDA, SOD, CAT, and thiol) in sensitized animals were assessed which reflected inflammatory, immunological, and oxidant changes in the lung of sensitized animals rather than systemic changes published in our previous paper. Sarkar et al. [7] also studied the effect of *C. longa* on total and differential WBC, nitrite, MDA, and myeloperoxidase (MPO) levels and percentage of intact and granulated mast cells in BALF, total protein in lung tissue homogenate, and serum IgG in sensitized animals. However, the inflammatory indices (PLA_2 and TP) as well as the important immunological indices such as IgE, IL-4, IFN-γ, and IFN-γ/IL-4 ratio which reflect Th1 and Th2 activities were not measured in the mentioned study. In addition, in the present study, the effect of three concentrations of *C. longa* extract (0.75, 1.5, and 3 mg/ml equal to 0.15, 0.3, and 0.6 mg/kg/day) added to the drinking water of animals throughout 21 days of sensitization period was evaluated. However, in the study of Sarkar et al. [7], the effect of oral administration of 100 mg/kg of the extract, twice diurnal at 8.00 A.M. and 18.00 P.M., was examined which was much higher than the administered dose in our study.

The use of ELISA kits to detect inflammatory mediators and cytokines levels in the BALF appears to be a limitation of the current investigation. However, they do not involve any quality control samples, because the results of various groups were compared. The inclusion of a quality control sample would have no effect on the overall results. As seen in Figure 5, the variation in the obtained values was similarly quite minimal.

Concentration-dependent effects of the extract of *C. longa* on all investigated variables were observed in the present study. The effect of its two higher concentrations was higher than its low concentration. In addition, the effect of high concentration of *C. longa* was also higher than its medium concentration. The concentration-dependent effect of the extract of *C. longa* on total and differential WBC counts, inflammatory mediators, and immunological, antioxidant, and oxidant biomarkers in OVA-sensitized rats also supported antioxidant, anti-inflammatory, and immunomodulatory properties of this plant.

The results of the current study also indicated comparable or even higher effects of the extract of *C. longa* compared to those of dexamethasone. The effects of all concentrations of the extract of *C. longa* on IFN-γ, its two higher concentrations on total WBC, IL-4, and IFN-γ/IL-4 ratio, and its high concentration on eosinophils, lymphocytes, and monocytes percentages and BALF levels of TP, IgE, and thiol were higher than the effect of dexamethasone. Based on our results, a more specific immunomodulatory effect was observed for *C. longa* extract in comparison with dexamethasone. In asthmatic rats, treatment with *C. longa* extract lowered IL-4 levels while it increased IFN-levels and the IFN-/IL-4 ratio, showing decreased Th2 but higher Th1 activity and improved Th1/Th2 balance. Treatment with the extract of *C. longa* reduced IL-4 but increased IFN-γ level and IFN-γ/IL-4 ratio, indicating decreased Th2 but increased Th1 activity and enhanced Th1/Th2 balance in asthmatic rats. However, dexamethasone treatment reduced both IFN-γ and IL-4 and did not change IFN-γ/IL-4 ratio and Th1/Th2 balance. These results also indicated a possible preventive therapeutic effect for *C. longa* on asthma which was more potent and specific compared to that of dexamethasone.

5. Conclusion

The novel finding of the current investigation was that *C. longa* had a more specific immunomodulatory effect on Th1/Th2 balance in sensitized rats when compared to dexamethasone. In addition, the current study shows that *C. longa* has anti-inflammatory, immunological, and oxidant-antioxidant properties in an animal model of asthma. The findings of this study imply that *C. longa* therapy reduces airway inflammation by restoring the oxidant-antioxidant equilibrium. In addition, *C. longa* immunomodulatory properties may help to reduce lung inflammation.

As a result, our findings could point to complicated interactions between *C. longa* antioxidant, anti-inflammatory, and immunoregulatory effects in the treatment of asthma. The current data, along with those from prior studies, demonstrate that *C. longa* has anti-inflammatory, immunomodulatory, and antioxidant properties in sensitized rats, implying that it could be used to treat asthma. However, more research is needed to determine how *C. longa* affects asthmatic patients.

Finally, our findings showed that *C. longa* had a protective effect on lung inflammation, oxidative stress, and immunological indicators in sensitized animals. At the concentrations tested, *C. longa* had anti-inflammatory and antioxidant effects that were equivalent to or even better than dexamethasone, but its immunomodulatory action was

more specific, resulting in an enhanced Th1/Th2 balance. These findings imply that *C. longa* has therapeutic promise in the treatment of asthma.

Data Availability

The data used to support the findings of this study are included within the supplementary information file(s).

Conflicts of Interest

The authors disclose no conflicts of interest.

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Research Article

Inhibitory Effect of *Phellinus baumii* Extract on CFA-Induced Inflammation in MH-S Cells through Nuclear Factor-κB and Mitogen-Activated Protein Kinase Signaling Pathways

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Phellinus baumii is a mushroom utilized as a traditional medicine for a wide range of human ailments, including diabetes, hypertension, hypercholesterolemia, and cancer, in Asia. The purpose of this study was to find out whether *Phellinus baumii* extract (PBE) could reduce inflammation caused by coal fly ash (CFA) in alveolar macrophages (MH-S). The anti-inflammatory effect of PBE was evaluated by measuring the nitric oxide (NO) concentration after the onset of CFA-stimulated inflammation in MH-S cells. Polymerase chain reaction (PCR) was used to examine inflammatory gene expression. Western blotting and immunofluorescence (IF) studies were used to investigate the inflammatory mechanism in MH-S cells. According to our results, the PBE suppressed CFA-induced NO generation in the MH-S cells dose-dependently. Furthermore, PBE inhibited the proinflammatory mediators and cytokines generated by exposure to CFA, including cyclooxygenase 2 (COX-2) and inducible NO synthase (iNOS), interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α). Real-time PCR was also used to determine the inhibiting effect of the PBE on proinflammatory factors such as COX-2, iNOS, IL-1β, IL-6, and TNF-α. Moreover, Western blot was used to assess the effects of the PBE on the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in the CFA-stimulated MH-S cells. The suppressive effect of the PBE on phosphorylated (p)-NF-κB translocation was also investigated using IF analysis. This study showed that the PBE suppressed the CFA-induced inflammation in the MH-S cells by suppressing the NF-κB and MAPK signaling pathways, which suggests its potential usefulness in reducing lung inflammation.

1. Introduction

Inflammation is an important aspect of the immune system's defense against damaging stimuli [1, 2]. Dysregulated inflammation is linked to various chronic disorders [3, 4]. Macrophages play an important role in the operation of inflammatory processes, primarily by producing proinflammatory mediators and cytokines such as nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2), interleukin 1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) [4]. Thus, the inhibition of the proinflammatory macrophage activation is considered a vital approach for treating inflammatory disorders.

Particulate matter (PM) in contaminated air is becoming a critical source of health issues [5]. Chronic PM exposure has been linked to chronic inflammatory disorders, particularly severe lung diseases such as chronic respiratory diseases, chronic obstructive pulmonary diseases, asthma, and several types of lung cancer [6–8]. Many pathological and clinical signs of airway inflammatory disorders are linked to excessive production of proinflammatory mediators and cytokines [9, 10]. Alveolar macrophages (MH-S) are

immune cells in the immunological regulatory system that are found in the pulmonary alveoli and responsible for upregulating inflammatory mediators caused by exposure to coal fly ash (CFA).

Phellinus baumii, a wild fungus, has traditionally been used as healthy food or a folk tonic in East Asia because of its multiple physiological functions, including blood lipid level-lowering, antitumor, antiinfluenza, and antioxidation capacities, and DNA damage-protecting, immune-stimulating, and antidiabetic activities [11–13]. Polysaccharides, polyphenols, and flavonoids, which have a wide spectrum of health benefits and biological activities, have been reported as the principal bioactive ingredients of the *Phellinus* fungus in modern medical research [14–16]. The *Phellinus* fungus includes various yellow polyphenolic chemicals, such as hispidin, that have demonstrated substantial biological effects and have been used to treat diabetes, bacterial, and viral infections, ulcers, and cancer, according to prior studies [11, 17].

Using NO and cell viability assays, as well as the mRNA and protein expressions of proinflammatory cytokines and mediators, we evaluated the anti-inflammatory effects of the *Phellinus baumii* extract (PBE) on CFA-stimulated MH-S cells.

2. Materials and Methods

2.1. Chemicals and Reagents. RPMI medium for MH-S cells culture, 10% of fetal bovine serum (FBS), 1% of penicillin–streptomycin, Dulbecco's phosphate-buffered saline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), coal fly ash (CFA), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA); oligo-dT and iNOS, COX-2, IL-1β, IL-6, and TNF-α primers (Bioneer, Daejeon, Republic of Korea); TRIzol reagent (Invitrogen, CA, USA); proprep (iNtRON Biotechnology, Republic of Korea); bovine serum albumin (BSA) (Thermo Fisher Scientific, Republic of Korea); primary antibodies including phosphorylated (p)-IκB, p-NF-κB, and NOD-like receptor protein 3 (NLRP3), phosphorylated extracellular signal-regulated kinase (p-ERK), total (T)-ERK, phosphorylated c-Jun N-terminal kinase (p-JNK), T-JNK, p-p38, T-p38, β-actin, and horseradish peroxidase-linked secondary antibody were used (Cell Signaling Technology, Danvers, MA, USA). A secondary antibody was used for immunofluorescence (IF) (Alexa Fluor 555, IgG Fab2, Molecular Probes). Other reagents were obtained from Sigma-Aldrich.

2.2. Preparation of the Phellinus baumii Extract (PBE). We purchased *Phellinus baumii*, which we ground into a coarse powder. We next extracted in 70% ethanol for 24 h using an extractor at 60°C, followed by concentration using the rotary evaporator under reduced pressure. To make a fine powder, the crude extract was frozen overnight at −70°C and lyophilized using a freeze dryer. Finally, the dried extract was ground into a fine powder. During the experiment, the powder was dissolved in DMSO. The concentrate was partitioned using hexane and water, and the hexane-soluble fraction was discarded. The water-soluble fraction was extracted with ethyl acetate. The ethyl acetate-soluble fraction contained yellowish polyphenols of the styrylpyrone class and various oils. Oils were eliminated by washing with chloroform. The remaining polyphenol cluster was dried and powdered for further experiments [18].

2.3. Cell Culture and Treatment. The MH-S macrophages were cultured in the RPMI medium and supplemented with 10% heat-inactivated FBS and 1% antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin), according to our previously described method [19]. The cells were then incubated at 37°C with 5% CO_2 in a humidified incubator.

2.4. Nitric Oxide Assay. The Griess reagents A and B were used to measure NO concentration in accordance with our previous study [7, 19]. In a 24-well plate, MH-S cells were seeded and cultured for 18 h with or without CFA (2.5 μg/mL) and PBE (12.5, 25, 50, and 100 μg/mL) at the doses indicated. 100 μL of Griess reagents were mixed with 100 μL of cell culture supernatants and incubated for 10 min at room temperature. On a microplate reader, the absorbance at 540 nm was measured (VersaMax, Molecular Devices, CA, USA).

2.5. Cell Viability Assay. A cell viability experiment was performed as stated using a MTT reagent at 100 μL/well in the culture medium to test the cytotoxicity, as described previously [7, 19]. The supernatants were removed after 2-3 h of incubation at 37°C in 5% CO_2. The wells were filled with DMSO (100 μL/well) and incubated for 10 min at room temperature with shaking. Finally, the absorbance was measured at 560 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

2.6. Reverse Transcription Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction. Using previously described methods, a polymerase chain reaction (PCR) analysis was done [7, 19]. PBE (12.5, 25, 50, and 100 μg/mL) was applied to cells at the doses indicated for 30 min, followed by 18 h of CFA stimulation (2.5 μg/mL). The RNA was extracted from the cells using the TRIzol reagent.

Two micrograms of total RNA were annealed with oligo-dT at 70°C for 10 min and cooled for 10 min on ice before reverse transcription (RT) in 20 μL of reaction mixture at 42°C for 90 min on a thermocycler. To inactivate the reverse transcriptase, the reaction was stopped at 95°C for 5 min. In a PCR premix, cDNA produced from an RT reaction was used to perform a RT-PCR (Bioneer). On 1% agarose gel stained with ethidium bromide, the PCR products were electrophoresed. ImageQuant LAS 500 was used to visualize the band (GE Healthcare Life Sciences, Republic of Korea). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene. SYBR green was used in the real-time PCR. The primer sequences for RT-PCR and real-time PCR are given in Table 1.

Table 1: Primers were used for reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis.

RT-PCR	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGCCTCGTGGCTTTGG
COX-2	CACTACATCCTGACCCACTT	ATGCTCCTGCTTGAGTATGT
IL-1β	CTGTGGAGAAGCTGTGGCAG	GGGATCCACACTCTCCAGCT
IL-6	GTACTCCAGAAGACCAGAGG	TGCTGGTGACAACCACGGCC
TNF-α	TTGACCTCAGCGCTGAGTTG	CCTGTAGCCCACGTCGTAGC
GAPDH	CACTCACGGCAAATTCAACGGCAC	GACTCCACGACATACTCAGCAC
Real-time PCR		
iNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
COX-2	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
IL-6	TCCAGTTGCCTTCTTGGGAC	GTGTAATTAAGCCTCCGACTTG
TNF-α	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
GAPDH	CACTCACGGCAAATTCAACGGCAC	GACTCCACGACATACTCAGCAC

2.7. Western Blot Analysis. Western blotting was performed with several changes, as described previously [4, 19]. Proteins were isolated from cells, concentrations were determined, and samples were prepared in sodium dodecyl sulfate (SDS) and heated for 5 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the samples. The proteins were deposited to the poly (vinylidene fluoride) membranes and blocked for 1 h at room temperature with skim milk (5%). The membranes were washed with washing buffer, tris-buffered saline with Tween (TBST) three times for 10 min each time, and treated overnight at 4°C with the primary antibodies (1 : 1000), namely, p-IκB, p-NF-κB, NLRP3, p-JNK, T-JNK, p-ERK, T-ERK, p-p38, T-p38, and β-actin. Furthermore, the membranes were washed with TBST three times for 10 min each time and then incubated with horseradish peroxidase-labeled secondary antibodies (1 : 3000) for 1 h before rinsing with TBST three times for 10 min each. Enhanced chemiluminescence (ECL) solutions A and B (1 : 1 ratio) were used to detect the protein bands in Imager LAS 500 (General Electrics, Boston, MA, USA).

2.8. Immunofluorescence Analysis. The immunofluorescence (IF) experiment was performed as described previously [4, 19]. The MH-S cells were rinsed with DPBS and fixed for 10 min in 4% paraformaldehyde. The cells were also permeabilized for 10 min with 0.2% triton X-100 in TBS (TBST) and rinsed three times with TBST for 5 min each time. The samples were blocked for 1 h with 2% BSA before adding rabbit anti-p-NF-κB (primary antibody) overnight at 4°C. MH-S cells were rinsed three times with TBST for 5 min each time. The samples were incubated for 1 h in the dark with a secondary antibody and then washed three times with TBST before being mounted with a Prolong Gold Antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Cell Signaling Technology) to observe the nuclei and examined using confocal microscopy (LSM700, Carl Zeiss, Jena, Germany).

2.9. Statistical Analysis. The data are represented using the mean and standard error of the mean. One-way analysis of variance was used to establish the statistical significance. The statistical analysis results were considered significant at $^{*}p$ values < 0.05, $^{**}<0.01$, and $^{***}<0.001$ in comparison to the CFA only group and at $^{\#}p$ values < 0.001 in comparison to the basal group.

3. Results

3.1. Phellinus baumii Extract Protected against Coal Fly Ash-Induced Nitric Oxide (NO) Generation and Cell Death in the Alveolar Macrophage Cells. NO is a key mediator in the inflammatory process, and its overproduction contributes to the development of inflammatory disorders. The Griess reaction method was used to quantify the NO levels in the murine MH-S in response to CFA stimulation in this investigation. The NO induction was potently reduced by PBE dose-dependently (Figure 1(a)).

Cell vitality was determined using the MTT test, and the results showed that the PBE had no effect on cell toxicity compared with the basal group at the different concentrations used (Figure 1(b)). These findings showed that PBE suppressed the NO generation dose-dependently and that the dosages used were not cytotoxic.

3.2. Suppressive Effect of Phellinus baumii Extract on Coal Fly Ash-Induced Proinflammatory Cytokines in the Alveolar Macrophage Cells. The levels of the CFA-induced proinflammatory factors were reduced in MH-S after 30 min of pretreatment with PBE. To investigate the anti-inflammatory properties of the PBE, the mRNA expression of iNOS and COX-2, IL-1β, IL-6, and TNF-α were examined using RT-PCR. Levels of the proinflammatory factors were shown to be reduced dose-dependently (Figures 2(a)–2(f)). This result revealed that the PBE inhibited CFA-induced production of inflammatory cytokines and lowered the mRNA levels significantly.

3.3. Phellinus baumii Extract Ameliorated the Coal Fly Ash-Induced mRNA Expressions of Proinflammatory Cytokines in the Alveolar Macrophage Cells. To validate the RT-PCR results for proinflammatory factors in the MH-S cells, the mRNA expressions of proinflammatory mediators and

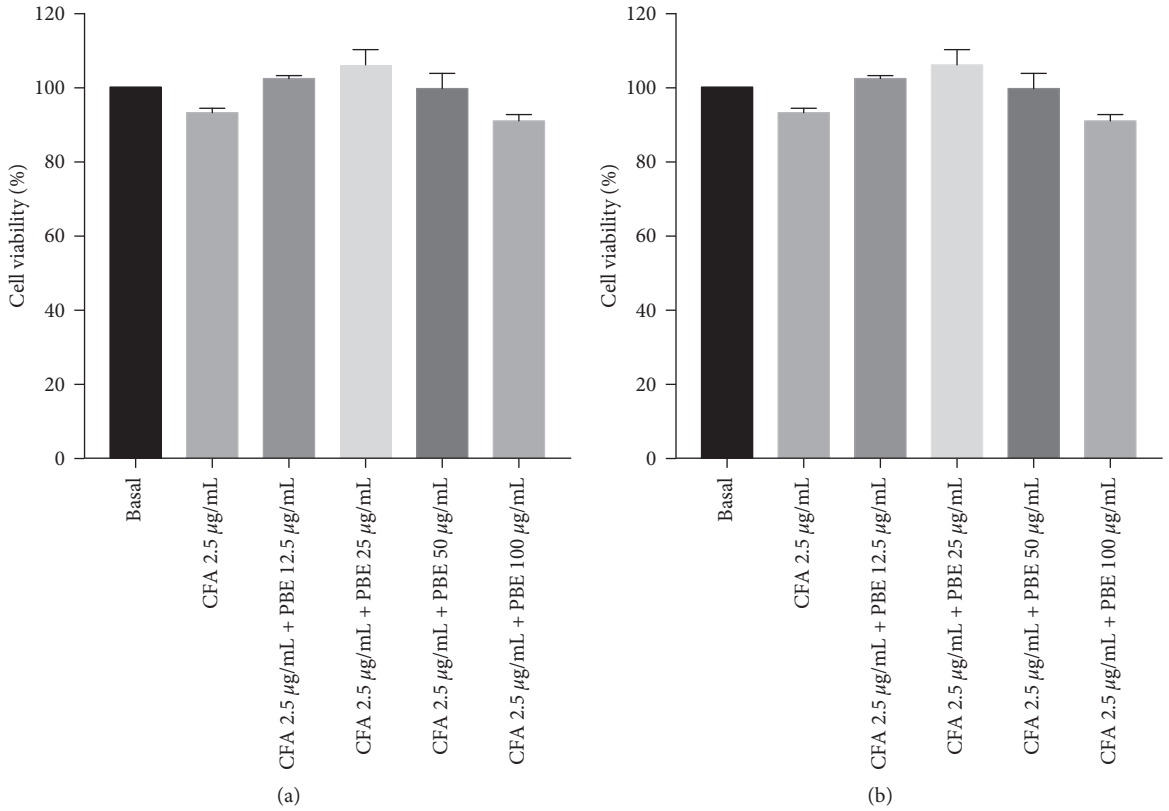

Figure 1: Effect of *Phellinus baumii* extract (PBE) on coal fly ash (CFA)-stimulated nitric oxide (NO) generation and cell viability in MH-S macrophages. (a) The cells are divided into six groups, namely, the control (basal), CFA only (2.5 μg/mL), and CFA with PBE (12.5, 25, 50, and 100 μg/mL) groups. The cells are treated with the abovementioned PBE concentrations for 30 min prior to the CFA treatment and incubated for 18 h. The NO level is determined using the Griess reagent method. (b) The MTT reagent method used to perform the cell viability experiment. A 24-well plate is used to seed the cells. All the values from the three independent experiments are expressed as standard error of the mean. $^{\#}P < 0.001$ in comparison to the basal group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in comparison to the CFA group.

cytokines were investigated, and those of proinflammatory factors were tested by real-time PCR. The PBE administration significantly reduced the mRNA expression levels of proinflammatory mediators and cytokines in a dose-dependent manner (Figures 3(a)–3(e)). The real-time PCR results demonstrated that PBE lowered proinflammatory factors in a concentration-dependent manner.

3.4. Phellinus baumii Extract Inhibits the Activation of the Nuclear Factor-κB (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) Signaling Pathways in the Coal Fly Ash-Treated Alveolar Macrophage Cells. It was hypothesized that the effect of PBE on NF-κB and MAPK signaling played a crucial role in inflammation. CFA activates the inflammatory pathway, and NF-κB and MAPK are critical pathways in the inflammatory cascade. Treatment with PBE considerably decreased the NF-κB phosphorylation and the inhibitor of kappa B (IκB) phosphorylation, whereas CFA markedly enhanced phosphorylation of NF-κB, a transcription factor and inhibitor of kappa B (IκB) in MH-S cells.

NLRP3 was also dose-dependently downregulated after treatment with PBE, especially at 100 μg/mL. In addition, the MAPK pathways, including p-JNK, p-p38, and p-ERK, were also significantly dose-dependently inhibited after treatment with PBE compared with treatment with CFA alone. These results suggest that the pretreatment with PBE significantly inhibited the CFA-induced NF-κB, IκB, NLRP3, ERK, JNK, and p38 activation in MH-S cells (Figures 4(a)–4(g)).

3.5. Phellinus baumii Extract Inhibited the Translocation of NF-κB in the Coal Fly Ash-Treated Alveolar Macrophage Cells. In CFA-stimulated macrophages, activated p-NF-κB translocation from the cytoplasm to the nucleus was examined using an IF assay to see if PBE's anti-inflammatory effects are mediated through signal transduction cascade of NF-κB. CFA treatment promoted the NF-κB translocation from the cytoplasm to the nucleus, but treatment with the maximum dose of PBE (100 μg/mL) dramatically inhibited p-NF-κB nuclear translocation in activated macrophages (Figure 5). Bay-11 was used as an NF-κB inhibitor. According to the immunostaining result, the anti-inflammatory activities of PBE were linked to its inhibiting properties of the NF-κB phosphorylation signaling cascade.

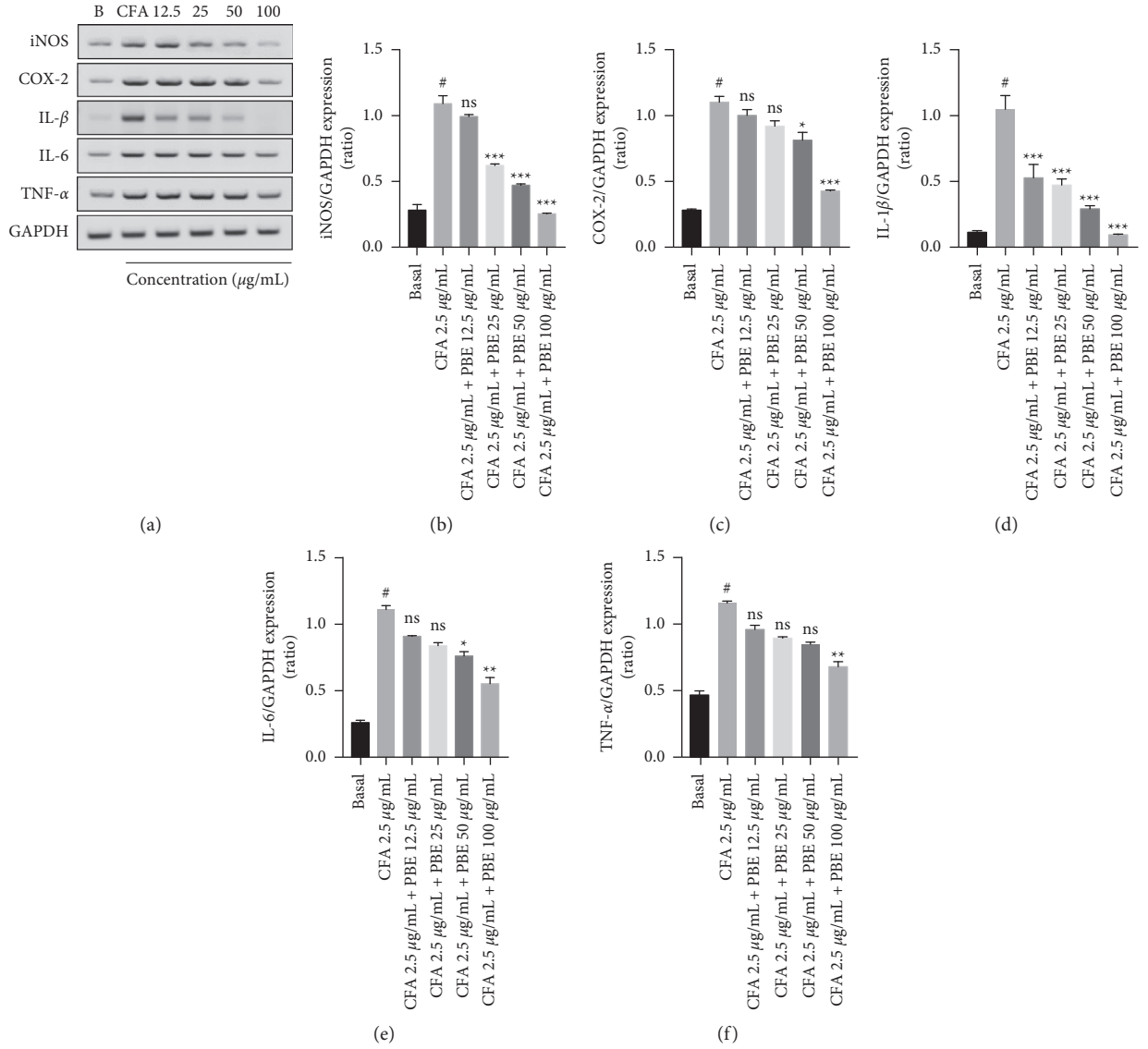

Figure 2: Effect of PBE on CFA-stimulated proinflammatory mediators and cytokines in MH-S macrophages analyzed using reverse transcription polymerase chain reaction (RT-PCR). (a) The mRNA levels of proinflammatory mediators (iNOS and COX-2) and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) measured after 18 h of CFA (2.5 μg/mL) incubation and GAPDH utilized as a housekeeping gene in RT-PCR. (b–f) The levels of protein expression densitometrically analyzed using ImageJ software. A 6-well plate is used to seed the cells, and the PBE doses of 12.5, 25, 50, and 100 μg/mL are used. All the values from three independent experiments are expressed as standard error of the mean. $^{\#}P < 0.001$ in comparison to the basal group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in comparison to the CFA group.

4. Discussion

As medicinal herbs have been widely studied for its anti-inflammatory properties [20, 21], we desire to investigate effective medicinal herbs that have potent medicinal properties. *Phellinus baumii* has long been used as herbal medicine in Asia, particularly China, Korea, and Japan for the treatment of a variety of ailments, including diabetes, hypercholesterolemia, and most notably cancer [11, 22]. Earlier studies showed that PBE can block NF-κB, transcription factor, which is a crucial regulator in the inflammatory cascade. The effects of *Phellinus baumii* on CFA-activated inflammation in the alveolar macrophage cell line were investigated in this work.

The extremely high production of NO related to iNOS synthesis is involved in the inflammatory process [23, 24]. iNOS plays a vital role in releasing NO during the pathophysiology of inflammatory diseases [25, 26]. Moreover, COX-2 is also stimulated by inflammatory stimuli during the inflammatory response [27, 28]. In our study, only the CFA-stimulated group showed upregulation of NO production compared with the basal group, whereas pretreatment with PBE reduced the NO production. The combined treatment

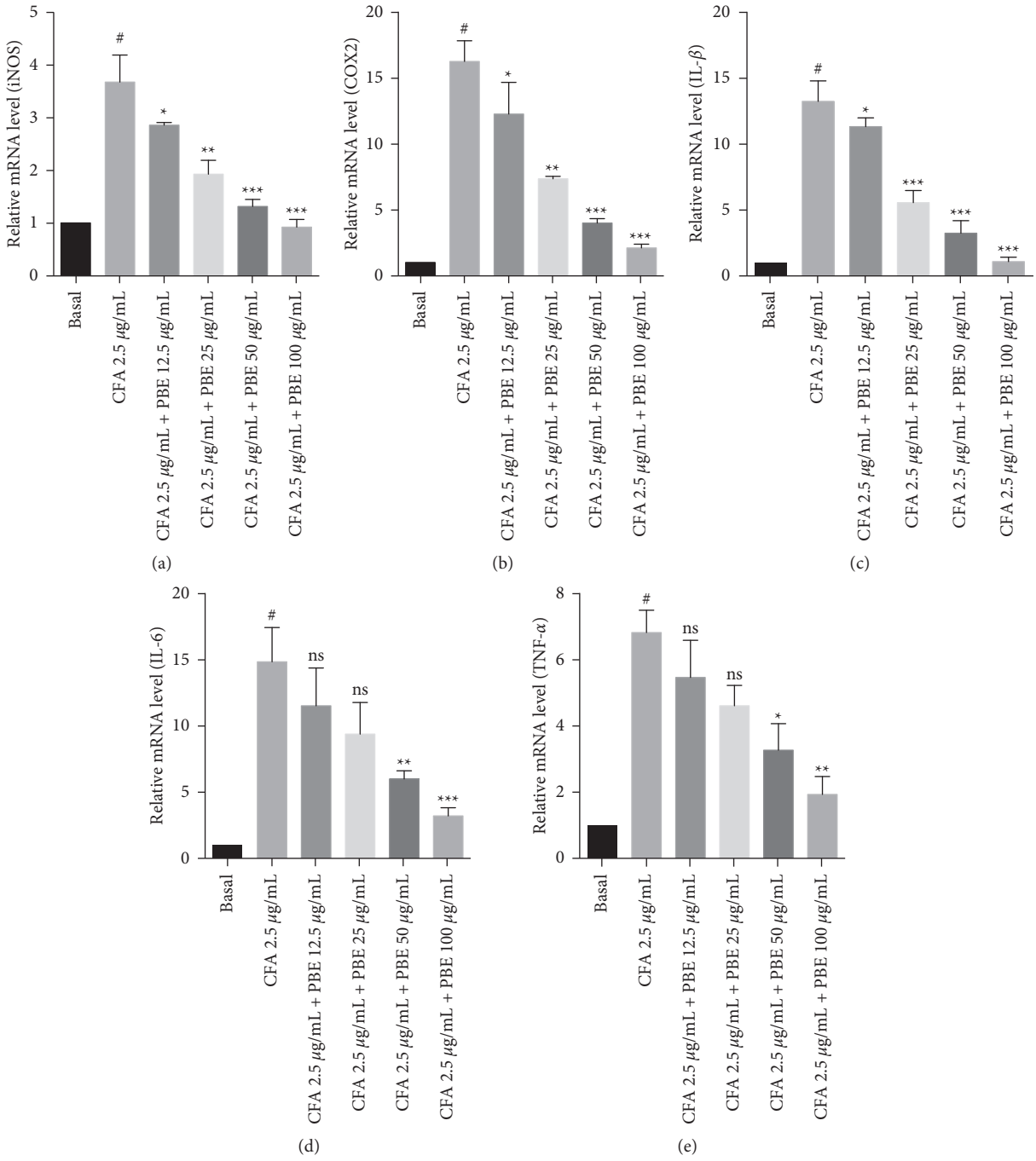

Figure 3: Effect of PBE on CFA-stimulated mRNA expression in MH-S macrophages measured using real-time polymerase chain reaction (PCR). (a–e) The mRNA levels of iNOS, COX-2, IL-1β, IL-6, and TNF-α are measured by quantitative real-time PCR after 18 h of CFA (2.5 μg/mL) incubation. GAPDH is used as a control gene. A 6-well plate is used to seed the cells, and PBE doses of 12.5, 25, 50, and 100 μg/mL are used. All the values from three independent experiments are expressed as standard error of mean. $^{\#}P < 0.001$ in comparison to the basal group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in comparison to the CFA group.

with PBE significantly decreased the NO induction (Figure 1(a)).

Endotoxins and cytokines prompted fast alterations in the NO gene expression throughout the inflammation process, which resulted in the de novo synthesis of iNOS and COX-2 pathways [24, 29]. The mRNA expression levels of the proinflammatory mediators such as iNOS and COX-2 and the proinflammatory factors such as IL-1β, IL-6, and TNF-α were upregulated in the CFA-treated group. By contrast, the combination treatment with PBE significantly downregulated the mRNA levels of the proinflammatory factors (Figures 2(a)–2(f)). These results suggest that PBE

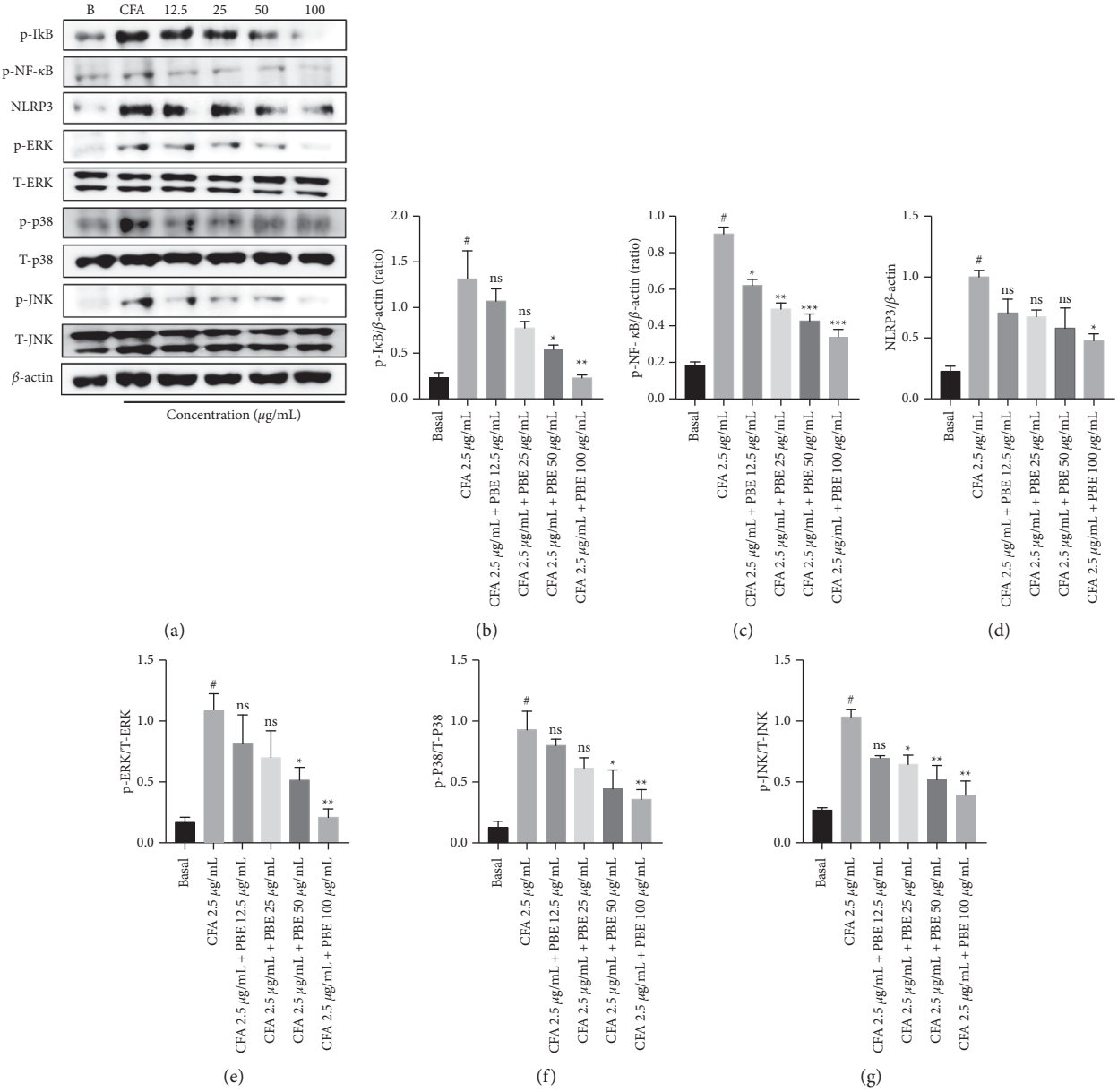

Figure 4: Effect of PBE on CFA-stimulated nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in MH-S macrophages. (a) The protein levels of p-IκB, phosphorylated NF-κB, and NOD-like receptor protein 3, and the MAPK (extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase) pathway investigated using Western blot after 18 h of CFA (2.5 μg/mL) incubation. As a loading control, β-actin was used. (b–g) The expression levels of protein were densitometrically analyzed using ImageJ software. A 6-well plate is used to seed the cells, and the PBE doses of 12.5, 25, 50, and 100 μg/mL are used. All the values from three independent experiments are expressed as standard error of the mean. $^{\#}P < 0.001$ in comparison to the basal group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in comparison to the CFA group.

has the potential to inhibit proinflammatory factors. Our results also support the previous findings that the iNOS and COX-2 expressions were upregulated in the CFA-stimulated group [19] and that treatment with PBE significantly reduced the protein levels of iNOS and COX-2 (Figures 3(a)–3(e)).

The NF-κB and MAPK signaling pathways were reported to be the key pathways for the inflammatory process [30–32]. The transcription factor NF-κB is a critical regulator in the inflammatory cascade [19]. The MAPK signaling pathway plays an important role in the inflammatory mechanism [33]. Therefore, we examined the anti-inflammatory mechanism of PBE in the macrophage cell line. The protein expression levels of p-IκB, p-NF-κB, NLRP3, p-ERK, T-ERK, p-p38, T-p38, p-JNK, and T-JNK were investigated using Western blotting. For the macrophage MH-S cell line treated with CFA, the activity levels of the NF-κB and MAPK signaling pathways were increased by the CFA treatment,

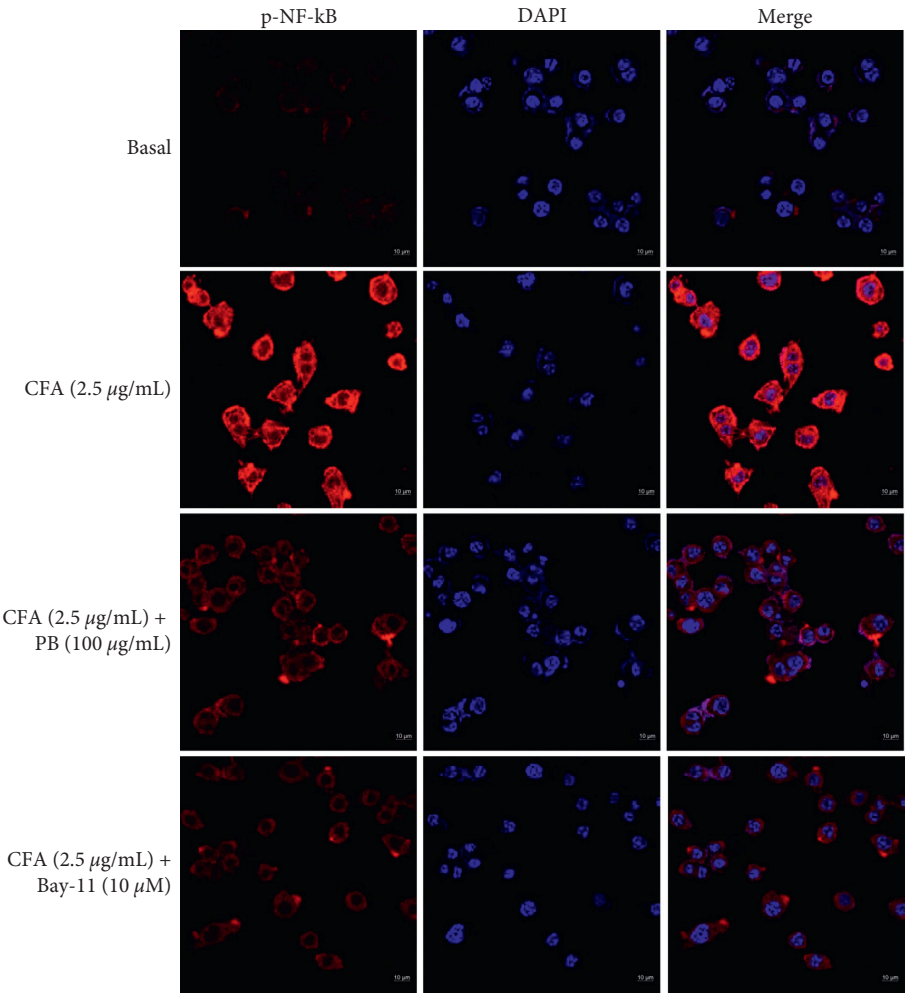

Figure 5: Effect of PBE on CFA-stimulated nuclear factor-kappa B (NF-κB) translocation in MH-S macrophages. The cells are seeded on a coated cover slip in a 6-well plate and grouped as follows: basal, CFA (2.5 μg/mL)-induced, CFA with PBE (100 μg/mL), and CFA with Bay-11, inhibitor of phosphorylated (p-NF-κB) groups. The cells are treated with PBE and Bay-11 (10 μM) for 30 min prior to the CFA treatment (2.5 μg/mL) and incubated for 18 h. Immunofluorescence staining is used to examine p-NF-κB nuclear translocation. To visualize the nuclei, the samples are mounted using a Prolong Gold Antifade reagent with DAPI (blue). Confocal microscopy (Zeiss) at ×400 original magnification is used to examine stained cells.

whereas treatment with PBE significantly suppressed the NF-κB and MAPK signaling protein expressions (Figures 4(a)–4(g)). On the basis of our findings, we hypothesized that PBE would decrease IκB and NF-κB phosphorylation, causing the proinflammatory factors to be suppressed.

Furthermore, IF staining revealed that CFA increased the p-NF-κB translocation in the nucleus, whereas PBE (100 μg/mL) and Bay-11 (10 μM) significantly decreased the p-NF-κB translocation from the cytoplasm to the nucleus (Figure 5). A similar result was also found in our previous study [19, 34]. Bay-11 has been reported to have an anti-inflammatory activity that inhibits the phosphorylation of IκB [35]. Our findings suggest that PBE inhibited CFA-stimulated inflammation by reducing the activity levels of NF-κB and MAPK signaling pathways (Figure 6). Only in vitro experiments were performed in this study. Large-scale animal excrement should be performed to reveal the specific mechanism of the action of PBE as an anti-inflammatory agent.

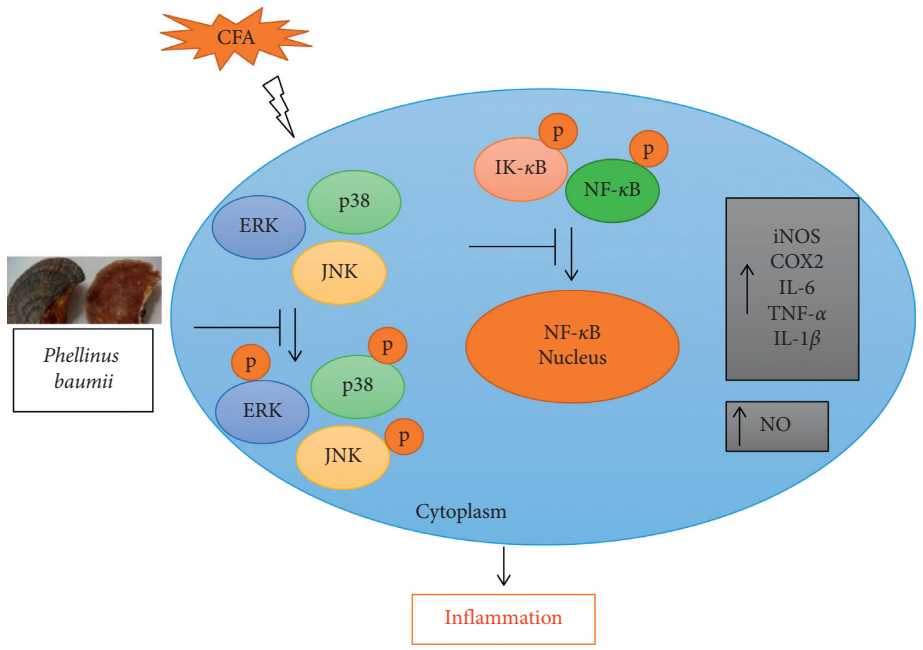

Figure 6: Mechanism of action of PBE in CFA-stimulated inflammation in MH-S macrophages.

5. Conclusion

In conclusion, the anti-inflammatory effects of PBE were established in the CFA-stimulated alveolar macrophages. The findings of this work add to our understanding of CFA-induced inflammatory responses and reveal that PBE may decrease proinflammatory factors expression in MH-S cells. According to our findings, the PBE has potential anti-inflammatory qualities for the regulation of inflammation and could be used as an herbal remedy in the prevention and treatment of numerous inflammatory disorders.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Effects of *Allium cepa* and Its Constituents on Respiratory and Allergic Disorders: A Comprehensive Review of Experimental and Clinical Evidence

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The health benefits of *Allium cepa* (*A. cepa*) have been proclaimed for centuries. Various pharmacological and therapeutic effects on respiratory, allergic, and immunologic disorders are shown by *A. cepa* and its constituents. Flavonoids such as quercetin and kaempferol, alk(en)yl cysteine sulfoxides including S-methyl cysteine sulfoxide and S-propyl cysteine sulfoxide, cycloalliin, thiosulfinates, and sulfides are the main compounds of the plant. *A. cepa* displays broad-spectrum pharmacological activities including antioxidant, anti-inflammatory, antihypertensive, and antidiabetic effects. Our objective in this review is to present the effects of *A. cepa* and its constituents on respiratory, allergic, and immunologic disorders. Different online databases were searched to find articles related to the effect of *A. cepa* extracts and its constituents on respiratory, allergic, and immunologic disorders until the end of December 2020 using keywords such as onion, *A. cepa*, constituents of *A. cepa*, therapeutic effects and pharmacological effects, and respiratory, allergic, and immunologic disorders. Extracts and constituents of *A. cepa* showed tracheal smooth muscle relaxant effects, indicating possible bronchodilator activities or relieving effects on obstructive respiratory diseases. In experimental animal models of different respiratory diseases, the preventive effect of various extracts and constituents of *A. cepa* was induced by their antioxidant, immunomodulatory, and anti-inflammatory effects. The preventive effects of the plant and its components on lung disorders induced by exposure to noxious agents as well as lung cancer, lung infection, and allergic and immunologic disorders were also indicated in the experimental and clinical studies. Therefore, this review may be considered a scientific basis for development of therapies using this plant, to improve respiratory, allergic, and immunologic disorders.

1. Introduction

Allium cepa L. *(A. cepa)* or onion species are used as vegetables and employed in traditional medicine as therapeutic agents [1–3]. Onion is a perennial plant that is cultivated in almost all countries, mainly in moderate climate regions such as Iran [4–6]. There are various onion varieties including white, yellow, purple, red, and green onions, which vary in color, and sweet and nonsweet onions differing in taste [4, 7, 8]. The stems of the plant are green, its leaves are hollow, its height can reach 1 m, and it has small white or purple flowers. The bulb of the plant which grows under the ground is used for medical or food purposes and as a spice with an exquisite odor and taste [9].

Onion bulbs have been used as a food, spice, and herbal remedy since ancient times by people around the world, and several therapeutic properties were described for this plant [6, 10].

A. cepa has been considered a famous herbal medicine in Ayurveda for several indications such as fever, dropsy, catarrh, and chronic bronchitis, in the forms of decoction, infusion, fresh juice, and raw, cooked, or roasted bulb [11]. The use of *A. cepa* species in the treatment of angina pectoris, dyspnea, dysentery, cough, and bronchial obstruction has been noted in Chinese pharmacopoeia [11]. In the ancient times, onion was used for various healing purposes in Egypt [12]. Furthermore, *A. cepa* tea has been used for treatment of fever, headache, cholera, dysentery, common cold, and arthritis in Chinese medicine [13]. The effect of *A. cepa* on respiratory diseases was also indicated in ancient Iranian traditional medical books [14–16].

Asthma is an inflammatory disease of the lungs that makes breathing difficult and limits physical activities. Various cells such as T cells, mast cells, basophils, macrophages, and eosinophils are involved in the inflammatory processes of asthma. Among these cells, higher numbers of eosinophils are a characteristic feature of asthma. Total white blood cell (WBC) and eosinophil counts were enhanced in sensitized animals and asthmatic patients. Therefore, attenuation of the inflammation is essential for the treatment of asthma [17].

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide that results in substantial social and economic burdens. COPD is a heterogeneous disease with both extrapulmonary and pulmonary components. Obstructive lung diseases are often diagnosed based on symptoms and decreased pulmonary function tests (PFT). Obstructive lung diseases are managed by avoiding triggers such as dust and smoking, use of bronchodilators to control symptoms, and suppression of lung inflammation [18].

Lung cancers are also among lung disorders which, despite advances in our understanding of risk factors involved, its development, and its immunologic control and treatment options, remains a leading cause of death. Tobacco smoking is the predominant risk factor for lung cancer development. The known risk factors for lung cancer include behavioral, environmental, and genetic risk factors, all of which play a part in tumor development. The low overall 5-year survival rate for lung cancer patients has only minimally changed in decades [19].

Acute respiratory infections account for 20–40% of outpatient and 12–35% of inpatient attendance in a general hospital. Upper respiratory tract infections including nasopharyngitis, pharyngitis, tonsillitis, and otitis media constitute 87.5% of the total episodes of respiratory infections. The vast majority of acute upper respiratory tract infections are caused by viruses. Common cold is mainly caused by viruses and does not require antimicrobial treatment unless it is complicated by acute otitis media with effusion, tonsillitis, sinusitis, and lower respiratory tract infection. Sinusitis is commonly associated with common cold. Most instances of rhinosinusitis are viral, and therefore, they resolve spontaneously without antimicrobial therapy. The most common bacterial agents causing sinusitis are *S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus*, and *S. pyogenes* [20]. Worldwide, tuberculosis is an important cause of pneumonia. Other pathogens such as viruses and fungi can cause pneumonia and severe acute respiratory syndrome and pneumocystis pneumonia. Pneumonia may develop complications such as lung abscess, a round cavity in the lung caused by the infection, or may spread to the pleural cavity [21].

Allergic conditions/disorders have increased during the last three decades all over the world due to changes in environmental factors including increased allergens, air pollution, and infection diseases [22]. Changes in foods and their amount in the diet may also contribute to increased risk of respiratory and allergic diseases. In addition, the interaction of environmental and genetic factors can affect the immune system and lead to the development of allergic diseases [23]. Serious allergic disorders include respiratory and skin allergies in which the immune system reacts to familiar allergens and reexposure to these agents leads to a massive secretion of allergy-related mediators which cause allergic symptoms [24].

Drugs that are currently used for the treatment of respiratory disorders may cause adverse effects and lack a high therapeutic efficacy; thus, new drugs should be developed for the treatment of these diseases [25]. Two types of drugs used for the treatment of inflammatory and obstructive respiratory diseases are relieving drugs that reduce airway obstruction and preventive drugs that suppress lung inflammation [26, 27]. Several adverse side effects were reported for drugs typically used in the treatment of asthma and allergic rhinitis such as antihistamines, decongestants, anticholinergic, and corticosteroids, including sedation, impaired learning and memory, and cardiac arrhythmias [25]. Therefore, therapeutic strategies should seek to decrease the side effects of the currently prescribed drugs. In fact, several safe natural therapies such as *Urtica dioica*, bromelain, quercetin (Qt), N-acetyl cysteine, and vitamin C have been introduced for treatment of the abovementioned disorders [28]. The antiallergic effect of polyphenols found in foods and plants on different disease models and clinical trials are shown; polyphenols have shown anti-inflammatory, antioxidant, and immunomodulatory effects and could modulate allergic sensitization by interaction with proteins

and inhibit mediator release [29]. Several studies also showed the preventive effect of derivatives from *A. cepa* such as Qt on respiratory disorders [30–32].

Treatments used against respiratory, allergic, and immunologic disorders with synthetic drugs do not fully cure these diseases and may cause various adverse side effects [33]. Therefore, using natural products such as some medicinal herbs, flavonoids, lactones, alkaloids, polysaccharides, diterpenoids, and glucosides, with immune-modulating and anti-inflammatory properties, may potentially help in treatment of respiratory, allergic, and immunologic disorders [34]. In fact, the effects of polyphenols on respiratory and allergic disorders such as atopic eczema, food allergy, and asthma were demonstrated [35]. Therefore, the effects of *A. cepa* and its constituents in respiratory, allergic, and immunologic disorders were reviewed in this article.

2. Constituents of *A. cepa*

A. cepa contains vitamins and minerals, sulfur amino acids, and a variety of secondary metabolites such as flavonoids (particularly flavonols and anthocyanin), phytosterols, and saponins [10]. Also, it is a rich source of phenolic acids, sulfur compounds (allicin), and various types of biological phytomolecules such as phenolic acids, thiosulfinates, anthocyanins, kaempferol, and glycosides [36, 37].

Onions contain two subgroups of flavonoids: (1) the anthocyanins that are responsible for red or purple color of some varieties and (2) flavanols such as Qt and its derivatives, which are responsible for the yellow varieties and brown color of the skin of onion. Another chemical group found in onion is the alk(en)yl cysteine sulfoxides (ACSOs), known as flavor precursors. The distinctive smell and taste of onions are due to the breakdown of ACSOs by the enzyme alliinase. Fructooligosaccharides are other types of phytochemicals in onions that mainly include inulin, kestose, nystose, and fructofuranosyl nystose [38].

In general, constituents of onions are classified as follows:

Polyphenolic substances: phenolic compounds in onions include protocatechuic, p-coumaric, ferulic acids, and catechol [39]. Onion phenolic acids are derived from benzoic acid or cinnamic acid. These phenolic acids help to create bitterness and aroma in the plant products [40].

Flavonoids*:* onion contains the basic flavonoids groups such as catechins (flavan-3-ols), leucoanthocyanidins (flavan-3,4-diols), flavanones, flavanonols, flavonols, and anthocyanidins. The predominant flavonol in onions is Qt which is present in free and bound forms and together with glycosides shows an antioxidant activity [41]. Other flavonoids in onions include luteolin and kaempferol [42]. The highest amount of flavonols is found in red onion, for red anthocyanins in the form of glycosides cyanidin, peonidine, and pelargonidine.

Ascorbic acid*:* ascorbic acid (vitamin C) is found in various amounts in a variety of vegetables and fruits. This water-soluble vitamin is reversible for the entire redox system [43, 44]. Vitamin C, Qt, and other active components of onions called isothiocyanates have anti-inflammatory effects [45].

Sulfur compounds: there are many organic compounds in onions, including sulfur, which is responsible for the unpleasant onion odors. The main ingredient in onion flavor is propylene-L-cysteine sulfoxide, which is annoying to some animals. Other sulfur compounds in onions include γ-glutamyl peptides, S-substituted cysteines, and cycloaline, which are nonvolatile and have no effect on onion taste [46]. Onion components and their biological activities are shown in Table 1. The chemical structures of the main constituents of the plant are presented in Figure 1.

3. Methods

Literature review was carried out by searching the databases PubMed, Scopus, and Web of Science using the following key terms: "*Allium cepa*," "onion," "flavonoid," "quercetin," "phenolic compounds," "therapeutic effects," "pharmacological effects," "allergic disorders," and "respiratory disorders" from 1984 to the end of 2020. Articles about the effects of *A. cepa* on respiratory and immunologic disorders, lung cancer, and lung infection written in the English language from 1984 to the end of 2020 have been incorporated in this article. The reference lists of the collected articles were also investigated to recognize further studies.

4. Traditional and Pharmacological Effects

Various pharmacological effects such as antidiabetic, antihyperglycemic, antiparasitic, antifungal, antimicrobial, antiplatelet, anti-inflammatory, antioxidant, and antispasmodic properties were reported for the extracts of *A. cepa* and its different constituents [28, 31, 42, 47–52]. The preventive effects of the extracts of *A. cepa* on the vascular and heart diseases [53], neurodegenerative and antidepressant disorders [8], and cataract formation as well as improving effects on kidney function were also reported [6, 54]. *A. cepa* has carminative and expectorant effects and could improve dysmenorrhea, vertigo, fainting, migraine, wounds, scars, keloids, pain and swelling after bee sting, bruises, earache, jaundice, and pimples [29]. *A. cepa* also showed antitumor activity [29] and could decrease the risk of stomach carcinoma [55] and inhibit proliferation of leukemia HL60 cells [56, 57].

The effect of *A. cepa* and its derivatives on respiratory diseases includes a relaxant effect on the tracheal smooth muscle (TSM) [58–61], a modulatory effect on the immune system [61], tracheal responsiveness and lung inflammation [17] in sensitized rats, antiasthmatic effects on a murine model of asthma [30], and antiasthmatic properties [62, 63]. The World Health Organization (WHO) also recommended using the *A. cepa* extract for the treatment of diseases including common colds, coughs, asthma, bronchitis, and allergic disease [64]. Onion animal extract showed

Table 1: Different constituents of *A. cepa*.

Major constituents	Other constituents	References
Water		[51]
Proteins		[51, 94]
Carbohydrates	Inulin, fructooligosaccharides, isorhamnetin-4-glucoside, galactose, glucose, and mannose	[51]
Vegetal hormone lectin	Glycoquinine	[13]
Steroids	Catechol, protocatechuic acid, thiocyanate, and thiopropiono aldehyde	[51, 94, 143]
Phytoestrogens	Coumestrol, zearalenol, isoflavones, and humulone	[51, 94, 143]
Vitamins	A, B complex, C, and E	[51, 94, 143]
Minerals	Selenium, phosphorus, iron, calcium, and chromium	[51, 94, 143]
Flavonoids	Quercetin, apigenin, rutin, myricetin, kaempferol, catechin, resveratrol, epigallocatechol-3-gallate, luteolin and genistein, quercetin aglycone, quercetin diglucoside, quercetin 4-glucoside, and isorhamnetin monoglycoside or kaempferol monoglycoside	[17, 51, 94, 144]
Organosulfuric compounds	Thiosulphinates, cepaenes, cysteine, S-methyl cysteine sulfoxide, diallyl disulfide, allyl methyl sulfide, allyl propyl disulfide, gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, S-propenyl cysteine sulfoxide, S-alk(en)yl cysteine sulfoxides, and S-allyl cysteine sulfoxide	[51, 145]
Allicin	Diallyl disulfide, diallyl trisulfide, and ajoene	[13, 51, 143, 146]
Phenolic compounds	Phenolics, phenolic acids, anthocyanins, and hydroxycinnamic acid	[42, 51]
Lipophilic antioxidants	Dialkyl disulfides, aglycones, anthocyanin, saponins, and fistulosin (octadecyl 3-hydroxyindole)	[41, 62]

antiasthmatic effect through leukotriene or thromboxane biosynthesis and histamine release inhibition [31].

The antiallergic potential of the extracts of *A. cepa* [31] and its flavonoid quercetin was reported in previous studies [32, 65]. It was also shown that the antiallergic potential of quercetin is similar to Chinese herbal formula (Food Allergy Herbal Formula) which inhibits anaphylaxis to peanuts in mice [66]. The anti-inflammatory and antiallergic properties of quercetin on respiratory and food allergies were also shown [67, 68]. Antiallergic [69], neuroprotective [70], anti-inflammatory, and antioxidant activities [71] were shown for derivatives of *A. cepa* including flavonoids, organosulfur, and phenols.

The polyphenol compounds present in onions showed stimulating effects on the immune system in the aging process [72, 73], and some phenolics in onions showed antiplatelet properties [74]. The antimicrobial effects of protocatechuic, p-coumaric, ferulic acids, catechol [7], and kaempferol [39] were also reported. Kaempferol also showed detoxifying, apoptotic, antineoplastic [75], anti-inflammatory, and antioxidant activities [76, 77].

The sulfur compounds possess antibacterial, antifungal, antitumor, and antilarval effects [78]. Therefore, onion sulfur compounds can be considered natural preservatives to control microbial growth [79]. Luteolin, Qt, and baicalein could inhibit the secretion of granulocyte macrophage colony-stimulating factor in human cultured mast cells, suppress the secretion of leukotrienes, prostaglandins D2, and histamine, and inhibit tumor necrosis factor- (TNF-) α and IL-6 in bone marrow-derived culture fluid cells [80]. Due to antioxidant ability and cholesterol level-controlling properties of flavonoids and Qt present in onion, this plant is used in prevention and treatment of cardiovascular diseases [81, 82]. The protective effect of Qt on oxidative stress in Alzheimer's disease and neurodegenerative disorders was also demonstrated [83]. In addition, onion flavonoids could suppress proinflammatory factors of hematoma and improve the symptoms of intracerebral hemorrhage by inhibiting the activation of microglia [84]. Therefore, the onion extract has proven antiallergic and anti-inflammatory effects mediated via diverse mechanisms.

5. Bronchodilatory Effect of *A. cepa* and Its Constituents and Relieving Effects of These Agents on Obstructive Pulmonary Disorders

5.1. Effects of the Plant Extracts and Essential Oil

5.1.1. Experimental Evidence. In a study, *A. cepa* extracts (AcE), (2, 4, 8, 16, 32, and 64 mg/ml) showed concentration-dependent relaxant effects on tracheal smooth muscle (TMS) of rats contracted by KCl or methacholine. There was no significant difference in the relaxant effects of AcE between nonincubated and incubated tissues with glibenclamide, atropine, chlorpheniramine, and indomethacin. EC50 values of AcE in TSM incubated with propranolol and diltiazem were significantly lower than nonincubated tissues. The relaxant effects of different concentrations of the AcE were not significantly different from those of theophylline. The concentrations of AcE and theophylline were significantly correlated with their relaxant effects. In TSM incubated with propranolol and diltiazem, concentration ratio minus one (CR-1) values were positive. The results showed a potent relaxant effect of the plant on TSM which was possibly induced by β2-adrenergic stimulation and/or calcium channel blockade. These findings suggest a possible bronchodilatory effect for AcE in obstructive pulmonary diseases [61].

Mandukhail et al. reported dose-dependent (3–30 mg/kg) reduction of carbachol (CCh), (1 mg/kg)-induced bronchoconstriction by a flavonoid-rich hydroacetone AcE peel, similar to the effect of aminophylline in rats. In guinea pigs also, the AcE (0.3–3 mg/mL) relaxed both CCh (1 μM) and high K^+-induced contraction of TSM concentration dependently and shifted the isoprenaline-induced relaxation concentration-response curves to the left, similar to effect of

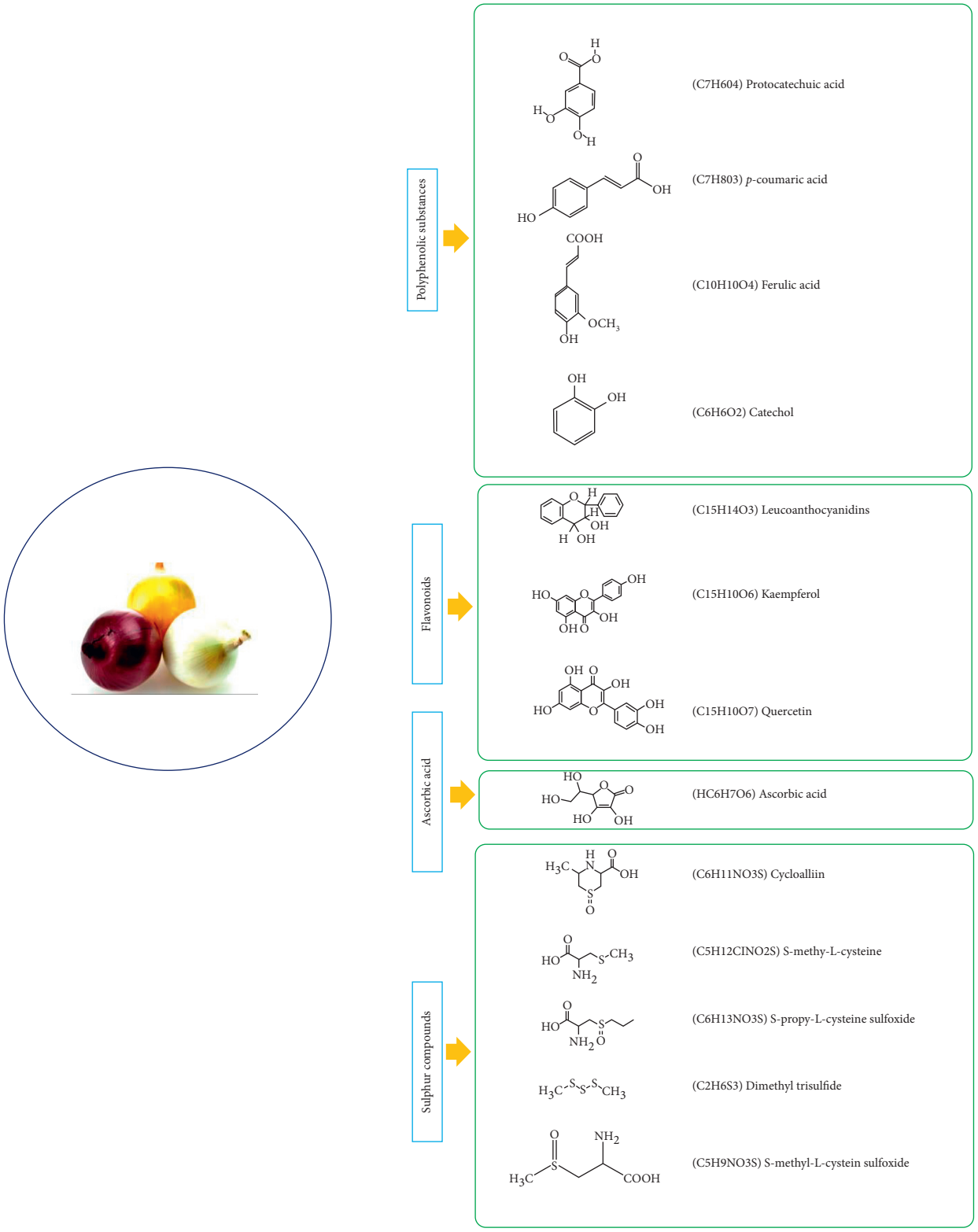

Figure 1: Chemical structure of the main constituents of *A. cepa*.

papaverine. The results indicated that the relaxant effect of the AcE on TSM is mediated through inhibition of Ca^{2+} channels and phosphodiesterase enzyme-like mechanism, suggesting red onion peel as a bronchodilatory agent in obstructive pulmonary diseases [85].

Benzyl-isothiocyanates (BITC, 15, 30, 75, and 150 mg/kg), a component of onion, also decreased bronchial obstruction dose dependently. In addition, ethyl-isothiocyanates and allyl-isothiocyanate similarly inhibited bronchial obstruction. However, no antagonistic effects of

ethanolic AcE 10 μl/ml given orally to the animals 30 min prior to allergen inhalation challenges on histamine- or acetylcholine (ACh-) induced bronchial obstruction were shown [86].

5.2. Effects of the Plant's Constituents

5.2.1. Experimental Evidence. The relaxant activities of various concentrations of Qt (3.5, 7.5, and 15 μg/ml) on TMS of A/J mice precontracted with CCh were reported [48]. It was shown that the ethyl acetate fraction of Qt (10 μM–1.0 mM) prevents Ca^{2+}-permeant L-type voltage-dependent Ca^{2+} channels (LVDCCs), short transient receptor potential channel 3 (TRPC3), and stromal interaction molecule (STIM)/Orai channels, leading to inhibition of precontraction of TSM in mice. In addition, ACh-induced contraction of TSM was inhibited by Qt. Therefore, Qt is able to inhibit Ca2+-permeant LVDCCs, TRPC3, and STIM/Orai channels that relax the precontracted TSM. These results suggest that Qt could be used to develop a new bronchodilator drug to treat obstructive lung disorders such as asthma and COPD [87].

In an *in vitro* study, Qt (100 nM-1 mM) acutely and concentration dependently relaxed TSM precontracted with ACh. Qt (50 μM) also markedly potentiated isoproterenol-induced relaxations of TSM. Qt directly mitigated phospholipase C activity, inositol phosphate synthesis, and intracellular calcium responses to Gq-coupled agonists. In an *in vivo* study, nebulized Qt (100 μM) also considerably attenuated methacholine-induced airway resistance. These results indicated that the bronchodilatory effects of QT were possibly mediated by selective inhibition of phosphodiesterases-4 (PDE_4), suppression of degradation of cyclic adenosine monophosphate, and increase in PKA signaling in TSM or through β-receptor stimulation [88].

The effects of Qt on ovalbumin- (OVA-) sensitized conscious guinea pigs and airway obstruction induced by histamine and ACh were examined using whole body plethysmography; results showed significant bronchodilation induced by Qt at 20 mg/kg. These results suggest the possible use of Qt for the treatment of airways obstruction because of its bronchodilatory effects *in vivo* and *in vitro* [89].

In a similar study, the relaxant effects of Qt on both CCh and electrical field stimulation- (EFS-) induced TSM precontraction were observed. The results also showed more prominent relaxant effects for Qt in TSM contracted by EFS than that contracted by CCh, suggesting a presynaptic effect for Qt in addition to the postsynaptic effect, as revealed by the inhibitory action of Qt on CCh-induced contractions. The inhibitory effect of Qt on contractions induced by EFS was not affected by phentolamine plus propranolol, tachykinin NK_1 and NK_2 receptor antagonists, and capsaicin treatment or by the proteolytic enzyme α-chymotrypsin. In contrast, the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester significantly decreased the inhibitory effect of Qt on contractions induced by EFS [59].

Concentration-dependent relaxant effects of Qt were shown on ACh or histamine-contracted human airways smooth muscle (HASM). In addition, K^+ and Ca^{2+} concentration-contraction curves were inhibited by incubation of HASM with increasing concentrations of Qt. Qt also enhanced the relaxant effects of isoprenaline or sodium nitroprusside concentration dependently. These findings indicated that the bronchodilatory effects of Qt are possibly mediated through increasing cyclic nucleotide levels and altering availability of Ca^{2+} to the contractile machinery [90].

In the sensitized guinea pigs to OVA, Qt 20 mg/kg administered 30 minutes before the contractile agents significantly inhibited airway contraction induced by cumulative doses of histamine or ACh, indicating the bronchodilatory effect of Qt on allergic asthma [60].

Overall, the experimental studies showed the relaxant effect of AcE and its constituent, Qt, on precontracted TSM induced by various smooth muscle contractile agents. The possible mechanisms of the relaxant effect of AcE or Qt on TSM are β2-adrenoceptors stimulation and/or inhibition of muscarinic and histamine H_1 receptors, calcium channel blocking, and phosphodiesterase enzyme mechanisms. These results suggest the possible bronchodilatory effects of the plant and its constituent on obstructive respiratory diseases but further clinical studies are needed to examine this effect in asthma, COPD, or other obstructive pulmonary disorders. The possible bronchodilatory effects of *A. cepa* and its constituents are shown in Table 2, and mechanisms shown to underlie these effects are presented in Figure 2.

6. Preventive Effects of *A. cepa* and Its Constituents on Asthma

6.1. Effects of the Plant Extracts and Essential Oil

6.1.1. Experimental Evidence. The AcE (35, 70, and 140 mg/kg b.w.) and dexamethasone (1.25 μg/mL) effects on oxidants, antioxidants, and immunological markers in the bronchoalveolar lavage fluids (BALF) of OVA-sensitized rats revealed concentration-dependent improvement of these markers in treated groups. In addition, the effect of *A. cepa* extract was similar to the effect of dexamethasone [10].

In a similar study, the adjuvant effects of AcE (150 and 300 mg/kg b.w.) and dexamethasone (1 mg/kg) on OVA-sensitized Wistar rats were examined. Eosinophil and lymphocyte in the blood and the BALF of the asthmatic group were significantly increased but decreased in the AcE-treated groups, indicating the reduction of cellular infiltration and lung inflammation of AcE-treated asthmatic rats [91].

Ghorani et al. also demonstrated that aqueous-alcoholic AcE (0.175, 0.35, and 0.7 mg/mL) and dexamethasone (1.25 μg/mL) administration to OVA-induced asthmatic rats during the sensitization period reduced tracheal responsiveness, lung inflammatory cells, and phospholipase A2 (PLA2) level in the BALF of the animals [17].

Table 2: The possible bronchodilatory effects of *A. cepa* and its constituents on the tracheal smooth muscle and its possible mechanisms.

Study type	Study design	Preparations	Dose	Effects	Ref.
Exp	Murine TSM contracted with Cch	*A. cepa* extract	10, 100, and 1000 μg/ml	Relaxing activity on TSM	[30]
	Rat TSM contracted with Mch and KCl	*A. cepa* extract	2, 4, 8, 16, 32, and 64 mg/ml	β2-Adrenergic stimulatory and calcium channel blockade mechanisms	[61]
	Rat trachea contracted with Cch	*A. cepa* peel	3–30 mg/kg	Inhibition of Ca^{2+} channels and phosphodiesterase enzyme-like mechanisms	[85]
	Animals 30 min prior to allergen inhalation	Ethanolic extracts	10 μl/1 ml, orally	Relation effect on TSM	[86]
	A/J mice precontracted with Cch	Qt	3.5, 7.5, and 15 μg/ml	Relaxing activity on TSM; inhibited Ca^{2+}-permeant LVDCCs, TRPC3, and STIM/Orai channels	[87]
	TSM of mice	Qt	100 nM-1 mM and 50 μM	Relaxant effect on TSM; PDE4 inhibition	[88]
	OVA-sensitized guinea pigs	Qt	20 mg/kg	Relaxant effect on TSM *in vivo* and *in vitro*	[89]
	Cch-induced TSM contractions	Qt	10^{-6}–3×10^{-4} M	Inhibited nitric oxide synthase; N^G-nitro-L-arginine methyl ester significantly reduced the effect of Qt	[147]

Ref.: references, Exp: experimental, Clin: clinical, TSM: tracheal smooth muscle, OVA: ovalbumin, TQ: thymoquinone, and Cch: carbachol.

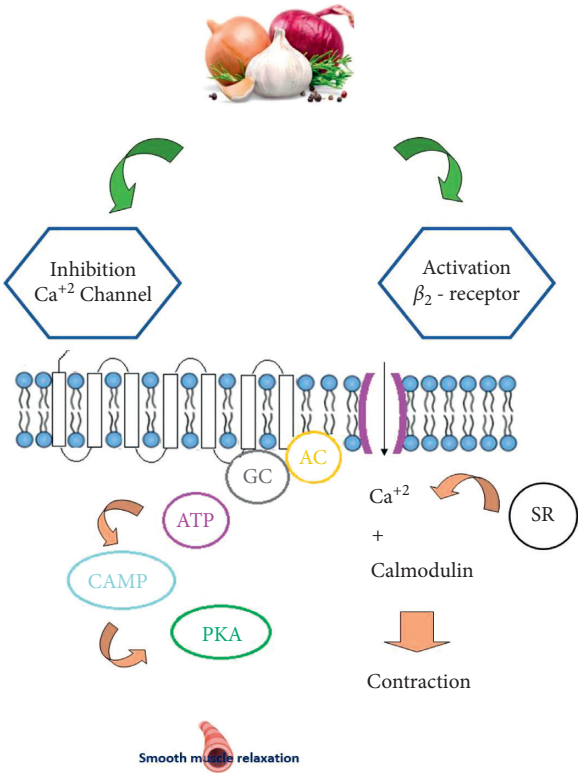

Figure 2: The possible mechanisms of the relaxant effect of *A. cepa* and its constituents on the tracheal smooth muscle.

6.2. Effects of the Plant's Constituents

6.2.1. Experimental Evidence. Antiasthmatic effects of the constituents of *A. cepa* were shown to be mediated through reduction of oxidative markers such as malondialdehyde (MDA), inflammatory mediators such as nuclear factor kappa B (NF-κB), prostaglandin D_2 (PGD_2), leukotrienes, and granulocyte macrophage-colony stimulating factor (GM-CSF), elevation in antioxidants such as superoxide dismutase (SOD), and suppression of T helper (Th) 2-type synthesis of cytokines such as IL-4 and IL-13 [22, 92].

In asthmatic mice, Qt treatment markedly reduced airway hyperresponsiveness and inflammatory cell numbers in the BALF, inhibited matrix metalloproteinase (MMP) 9 and GATA-3 mRNA levels in the lung tissues, and improved Th1/Th2 balance (decreased Th2 cytokines IL-4 and IL-5 but increased Th1 cytokine interferon gamma (IFN-γ)) [32].

The AcE and the constituents of onion, mainly Qt, decreased total and differential WBC in the blood and BALF of animals sensitized with OVA (an animal model of asthma). Oxidant markers such as MDA was reduced, but antioxidants including CAT and SOD were increased in asthmatic animals due to treatment with AcE and Qt. Serum and BALF levels of PLA2, NF-κB, PGD_2 leukotrienes, and GM-CSF were also decreased due to treatment with AcE and Qt. Treatment with the plant and its constituents also decreased tracheal responsiveness and lung pathological changes in the sensitized animals. Th2-type cytokine (such as IL-4 and IL-13) synthesis was decreased, but Th1 cytokine IFN-γ was increased and Th1/Th2 balance was improved in asthmatic animals treated with AcE and the constituents of the plant [93].

Other active components of onions including thiosulfinates and sulfines (sulfinyl disulfides) are able to activate cyclooxygenase and 5-lipoxygenase pathways which initiate eicosanoid metabolism. Thus, these constituents might be responsible for anti-inflammatory and antiasthmatic properties of the onion extracts [94].

Treatments with kaempferol attenuated the Th2-driven allergic airway disease in an experimental model of asthma by decreasing production of IL-5 and IL-13 and amelioration of airway hyperresponsiveness (AHR) induced by OVA challenge. Kaempferol also inhibited IgE-mediated release of proinflammatory mediators from human mast cells [95]. The preventive effect of Qt (3.5, 7.5, and 15 μg/ml) on cytokine levels in spleen cell culture supernatants showed a reduction in the production of inflammatory cytokines in

Blomia tropicalis- (BT-) sensitized A/J mice. Treatment with Qt (30 mg/kg) reduced the total number of cells in the BALF and erythropoietin (EPO) in the lung. These results demonstrate a reduction in the production of inflammatory cytokines and total number of cells in the BALF and EPO in the lungs by treatment with AcE or Qt [30].

6.2.2. Clinical Evidence. The results of several epidemiological studies suggest that consumption of Qt is beneficial for asthma therapy. Moreover, clinical trials on Qt have shown its ameliorative effects on symptoms related to asthma. Protective effects of Qt consumption on asthma incidence have been demonstrated by epidemiological and population-based case-control studies [36, 37, 96].

It was reported that diphenylthiosulfinate, a constituent of onion, inhibits the chemotaxis of human granulocytes induced by formyl-methionine-leucine-phenylalanine in a dose-dependent manner (0.1–100 mM) *in vitro*. The highest activity found for this agent was higher than that of prednisolone. Therefore, the anti-inflammatory properties of the onion extracts are related, at least in part, to its constituent, thiosulfinates, and this agent could be a candidate for the treatment of bronchial asthma [97].

Therefore, these results showed that *A. cepa* and its constituents could be considered possible preventive treatments for asthma. The ameliorative effect of Qt on asthma symptoms and its protective effect on asthma incidence were shown in epidemiological and population-based case-control studies. The preventive effects of *A. cepa* and its constituents on asthma are shown in Table 3.

7. Effect of *A. cepa* and Its Constituents on Lung Cancer

7.1. Effects of the Plant Extracts and Essential Oil

7.1.1. Experimental Evidence. Treatment with AcE (10 g/L) showed antiproliferative capacity, and there was an association between the concentration of the extracts and reduction of mitotic indices. Furthermore, the extract did not indicate antimutagenic and genotoxic activity. These effects might be related to the phenolic compounds found in the extracts of onion [98].

7.2. Effects of the Plant's Constituents

7.2.1. Experimental Evidence. Treatment of human lung cancer cell line NCI-H209 with Qt glucuronides decreased cell viability, dose and time dependently, but increased cell cycle and the proportion of cells in G2/M phase and subG0/G1 phase. Qt glucuronides also increased the expressions of cyclin B, Cdc25c-ser-216-p, and Wee1 proteins, indicating G2/M arrest. Decrease of mitochondrial membrane potential, release of cytochrome c, upregulation of Bax, downregulation of Bcl-2, activation of caspase-3, and cleavage of poly(ADP-ribose) polymerase were seen following Qt treatment, demonstrating the induction of apoptosis [99].

Treatment of A549 cells with Qt reduced cell viability, DNA synthesis, and Bcl-2 level but increased Bax, Bad, and Bcl-x(L), dose dependently. Moreover, Qt induced cleavage of caspase-3, caspase-7, and poly ADP-ribose polymerase (PARP), inhibited Akt-1 and p-Akt-1, and phosphorylated the extracellular signal-regulated kinase (ERK) and MEK1/2 in a dose-dependent manner. These findings suggest that Qt is able to induce apoptosis in A549 lung carcinoma cells [100].

The effects of *A. cepa* and its constituents on lung cancer were shown in several studies. Nicotine is a main toxic component of cigarette smoke that contributes to the development of lung cancer in smokers. In this regard, the protective effect of *A. cepa* oil as an antioxidant in nicotine-administered rats was examined. Treatment with *A. cepa* oil (100 mg/kg b.w. for 21 days) increased catalase (CAT) and SOD activity in the lung tissue of rats exposed to nicotine [101]. Another study also demonstrated that exposure of animals to nicotine led to emphysematous air spaces, with thickened interalveolar septa, massive congestion, extravasation of red blood cells, inflammatory cellular infiltration, and fluid exudate that were all improved by AcE administration. MDA level also decreased, but antioxidant marker (SOD and CAT) levels were increased due to treatment with AcE in rats [33].

7.2.2. Clinical Evidence. In nontumor lung tissue from 38 adenocarcinoma patients, Qt-rich food intake was negatively correlated with lung cancer risk which was not different between P450 or GST genotypes, gender, or histological subtypes and the correlation was stronger in smoker subjects (smoking >20 cigarettes a day). In addition, gene expression in the high Qt-rich food consumption group showed a higher upregulation of GSTM1, GSTM2, GSTT2, and GSTP1 but downregulation of specific P450 genes compared to the low consumption group. These data show an association between Qt intake, tobacco smoking, and lung cancer risk and a possible therapeutic effect of Qt on lung cancer [99].

Intake of a Qt-rich diet, in the tissue samples from 264 lung cancer cases (144 adenocarcinomas and 120 squamous cell carcinomas), differentiated miRNA expression profiles of the tumor suppressor let-7 family in adenocarcinomas. Carcinogenesis-related miR-146, miR-26, and miR-17 were also significantly differentiated due to Qt-rich diet. Among former and current smokers with adenocarcinoma, 33 miRs were also differentiated between highest and lowest Qt-rich food consumers. This study indicates the differential expression of biologically functional miRs in Qt-rich food consumers with adenocarcinoma and supports the therapeutic effect of Qt on lung cancer [102].

Overall, treatment with Qt affects different cancer cell lines through modulating cell viability and other molecular mechanisms indicating its therapeutic effect on lung cancer. Various clinical studies also support the effect of Qt on lung cancer. The effects of *A. cepa* and its constituents on lung cancer are summarized in Table 4.

Table 3: The preventive effects of *A. cepa* and its constituents on asthma.

Study type	Study design	Preparations	Dose	Effects	Ref.
Exp	Murine model of asthma	*A. cepa* extract	10, 100, or 1000 μg/mL, orally	Decreased recruitment of eosinophils and their activation in the lungs	[30]
	OVA-sensitized rat	*A. cepa* aqueous extract	150 and 300 mg/kg b.w.	Decreased cellular infiltration and lung inflammation	[17]
	OVA-sensitized rat	*A. cepa* aqueous extract	150 and 300 mg/kg	Decreased eosinophil and lymphocyte counts in blood and the BALF; inflammation was reduced	[91]
	BT-sensitized A/J mice	Qt	3.5, 7.5, and 15 μg/ml	Reduced total number of cells in the BALF; anti-inflammatory and immunomodulatory effects	[30]
	OVA-sensitized BALB/c mice	Isoquercitrin and Qt	15 mg/kg and 10 mg/kg	Decreased blood neutrophils, lung homogenate IL-5, and eosinophilic inflammation	[112]
	Airway epithelial cells	Qt	0.1–25 μM, orally	Decreased airway epithelial cell, IL-8, and MCP-1 expression by attenuating signaling through a PI-3 kinase/Akt/NF-κB pathway; inhibited chemokine expression	[136]
	OVA-sensitized guinea pigs	Qt	20 mg/kg	Decreased TR	[60]
Clin	Asthmatic patients	*A. cepa* extract	—	Improved clinical symptoms	[128]
	Asthmatic adult	*A. cepa* extract	15 mg/kg/day	Improved clinical symptoms; no effect on the Th17 cell count	[148]
	Asthmatic patients	Kaempferol	—	Reduced asthmatic attacks, improved FPT and ACT score	[126]
	Asthmatic patients	Thiosulfinates	500 mg	Improved FPT and ACT score; increased FEF 25–75% and FEV 1% and IFN-γ; decreased FeNO and IgE	[86, 142]
	Asthmatic patients	Kaempferol	0.90 ± 0.07 μg/L	Reduced asthmatic attacks; increased FVC and FEV1%	[149]

Ref.: references, Exp: experimental, Clin: clinical, TSM: tracheal smooth muscle, OVA: ovalbumin, TQ: thymoquinone, Cch: carbachol, Qt: quercetin, BALF: bronchoalveolar fluid, BT: *Blomia tropicalis*, [MCP]-1: monocyte chemoattractant protein, TR: tracheal responsiveness, FEV: forced expiratory volume, IFN-γ: interferon gamma, and FVC: forced vital capacity.

8. Effects of *A. cepa* and Its Constituents on Lung Infections

8.1. Effects of the Plant Extracts and Essential Oil

8.1.1. Experimental Evidence. The findings of Ziarlarimi et al.'s study showed that *Escherichia coli* (*E. coli*) was resistant to the aqueous extracts of AcE [103]. However, antibacterial activity of onion (50 mg/ml, twice daily for 7 days) has been shown, and it was indicated that the plant can be used in the treatment of bacterial diseases and as an immune booster to inhibit bacterial (*P. aeruginosa*) infections [104]. The decrement of gold nanoparticles synthesized with onion and inoculation of this combination affected *E. coli* in trypticase soy broth. Application of this combination to *E. coli* and incubation for a period of time caused cell lysis, showing antibacterial effect of the combination; thus, onion could be an effective candidate for sanitation of food and healthcare institutions [105].

The essential oil of *A. cepa*, at a concentration of 28.0 μL/100 mL, showed a fungicidal effect on the growth of *Aspergillus carbonarius*, *Aspergillus wentii*, *Aspergillus versicolor*, *Penicillium brevicompactum*, *Penicillium glabrum*, *Penicillium chrysogenum*, and *Fusarium spp*. In addition, the plant exerted an inhibitory effect on *Aspergillus niger* and *Penicillium aurantiogriseum* [106].

8.1.2. Clinical Evidence. In a clinical study, in viral flu patients with mild symptoms of cough, headache, and sputum production at the onset of disease, a simple home-based treatment (self-treatment) of an alternative approach with inhalation of a preparation of onion, garlic, or scallions improved all symptoms, suggesting application of these plants for treatment of mild virus-infected respiratory diseases at onset of the disease [107]. It was also indicated that *A. sativum* can combat COVID-19 infection by modulating immune system cells, reducing the production and secretion of proinflammatory cytokines, and affecting adipose tissue-derived hormone leptin with proinflammatory nature [108].

Treatment of *P. aeruginosa* with a high concentration of crude juices of garlic (*A. sativum*) and *A. cepa* showed low D-value, but the opposite was indicated for *S. aureus* [109].

8.2. Effects of the Plant's Constituents

8.2.1. Experimental Evidence. In several studies, the effect of Qt on microbial, viral, and parasitological lung infections was shown. Supplementation of intranasal viral instillation with oral Qt significantly reduced superoxide radicals and lipid peroxidation levels, the number of infiltrating cells, and lung morphological changes [110].

Table 4: The preventive effects of *A. cepa* and its constituents on lung cancer and lung infections.

Study type	Study design	Preparations	Dose	Effects	Ref.
Exp	Rats exposed to nicotine	*A. cepa* oil	100 mg/kg b.w., 21 days	Increased CAT and SOD activity in the lung tissue	[101]
	Mice	Qt	—	Decreased superoxide radicals and LPO; lung morphological changes	[110]
	Swiss albino male mice	Qt	—	Lowered the lipid peroxide levels	[111]
	Cell line NCI-H209	Qt glucuronides	—	Qt glucuronides inhibited proliferation through G2/M arrest of the cell cycle and induced apoptosis via caspase-3 cascade in the human lung cancer cell line NCI-H209	[99]
	Cat fish	*A. cepa* extract	1000 mg/ml	Antibacterial activity	[98]
	Mice	Ethanol extract	10 and 20 mg/ml	Antibacterial effect	[150]
	Mesocestoides corti infection in BALB/c mice	Qt	5.0–20.0 mg/kg, oral	Decreased peripheral blood eosinophilia and IgE hyperproduction	[135]
	Lung cancer patients	*A. cepa* extracts	10 g L^{-1}	Antiproliferative capacity; decreased mitotic indices	[98]
	Adenocarcinoma patients	Qt-rich food	—	Decreased lung cancer risk; upregulated GSTM1, GSTM2, GSTT2, and GSTP1; downregulated specific P450 genes	[102]
	Lung cancer patients	Qt-rich food	mg/100 g	Decreased carcinogenesis-related miR-146, miR-26, and miR-17 families	[151]
	DPT patients	Qt and QP	5 g in 100 mL saline	Reduction of the disease manifestation	[115]
Clin	Cell line NCI-H209	Qt glucuronides	—	Qt glucuronides inhibited proliferation through G2/M arrest of the cell cycle and induced apoptosis via caspase-3 cascade in the human lung cancer cell line NCI-H209	[99]
	Clinical study	*A. cepa* aqueous extract	—	Antibacterial effect on Gram-positive and Gram-negative bacteria; effect on A549 lung cancer cell lines	[100]
	Virus-infected patients	Inhaled onion, garlic extract	—	Improved all respiratory symptoms	[107]
	Clinical study	Vitamin C and Qt	30 or 40 mg/kg, 4 days	Treatment of respiratory tract infections with COVID-19	[112]
	Clinical study	Qt and NAC, nebulized	600 and 300 mg/mL for Qt and NAC	Decreased respiratory symptoms; antiviral in COVID-19 infection	[114]

Ref.: references, Exp: experimental, and Clin: clinical.

Treatment of influenza virus- (A/Hong Kong/8/68) infected Swiss albino mice with Qt decreased the lipid peroxide levels and formazan-positive cells in these mice [111]. Qt-loaded poly D,L-lactide-co-glycolide (PLGA) nanoparticles (PQTs) showed antibacterial activity on *E. coli* and *Micrococcus tetragenus* mediated by disrupting bacterial cell wall integrity dose dependently; the effect was more prominent on *E. coli* than *M. tetragenus*. In addition, the antibacterial activity in mice was also shown with the absence of lung pathological changes in treated animals with PQTs [97].

Treatment of influenza virus- (A/Udorn/317/72(H3N2)) infected mice with Qt increased GSH, SOD, and pulmonary concentrations of CAT but did not affect the fall in vitamin E level in the infected mice. Therefore, Qt may be of therapeutic value in protecting the lung injury due to oxidative stress induced by influenza virus infection [106].

8.2.2. Clinical Evidence. Treatment with vitamin C and Qt at doses of 30 or 40 mg/kg, BID, po, for 4 days was suggested for both prophylaxes in high-risk populations and for the treatment of COVID-19 patients as an adjunct to promising pharmacological agents such as convalescent plasma. In fact, Qt showed antiviral effects by interfering with virus entry and replication and protein assembly which were augmented by coadministration with vitamin C. Therefore, these two compounds could be promising candidates for both the prophylaxis and early treatment of virus respiratory tract infections, especially in COVID-19 [112]. It was also indicated that Qt inhibits various viral infection and replications at different stages without serious side effects and could be a promising drug for the treatment of the common cold [113].

The alleviating effects of antiviral, anti-inflammatory, and respiratory symptoms of Qt of nebulized 1 mL, Qt of 200 mg/mL, and 1 mL N-acetyl cysteine (NAC) (100 mg/mL, three times a day) were reported. Therefore, Qt formula could be recommended for further clinical study for COVID-19 and other viral pulmonary infections [114]. In patients with newly diagnosed destructive pulmonary tuberculosis, treatment with Qt and polyvinylpyrrolidone QP (5 g in 100 mL of 0.9% sodium chloride solution intravenously once a day for 10 days) resulted in quick reduction of the disease manifestation [115].

The reviewed papers indicated the effect of the extracts, essential oil, and the constituents of onion, mainly Qt, on viral, microbial, parasitic, and fungal infections in the lung. In experimental studies, the effect of onion on the lung infected with *E. coli* and *P. aeruginosa* was shown. The essential oil of *A. cepa* affected lung infections with various fungi including *Aspergillus carbonarius*, *Aspergillus wentii*, *Aspergillus versicolor*, *Penicillium brevicompactum*, *Penicillium glabrum*, *Penicillium chrysogenum*, *Fusarium spp*, *Aspergillus niger*, and *Penicillium aurantiogriseum*. Treatment with Qt improved influenza virus infection and its lung manifestation. Clinical studies showed beneficial effects of onion on symptoms of virus-infected flu including cough, headache, and sputum production. The effect of onion on the lung infected with *P. aeruginosa, S. aureus,* and *S. pneumonia* was also demonstrated. Treatment with Qt showed antiviral effects caused by interfering with virus entry and replication and protein assembly [116]. The effect of Qt on the treatment of COVID-19 patients was also suggested, and its effect on pulmonary tuberculosis was also demonstrated. The effects of *A. cepa* and its constituents on lung infections are summarized in Table 4.

9. Effects of *A. cepa* and Its Constituents on Allergic Disorders

The effects of *A. cepa* and its constituents on asthma were described in previous sections. The effect of the plant and its constituents on allergic and immunologic disorders is provided in this section.

9.1. Effects of the Plant Extracts and Essential Oil

9.1.1. Experimental Evidence. In the Mediterranean diet, as well as in other diets, *A. cepa* is widely used in raw or cooked form [117, 118]. This plant is used for the treatment of allergic or upper airway diseases worldwide [17]. *A. cepa* is regarded as a folk remedy in almost all traditional and herbal medicines. Research studies also support the efficacy of the plant and showed positive effects of *A. cepa* and its constituents on immunologic and allergic disorders in animal studies [17, 119].

In allergic rhinitis in BALB/c mice induced by intraperitoneal administration of OVA and challenged with intranasal instillation of OVA, topical administration of *A. cepa* extract reduced allergic symptoms. The levels of OVA-specific IgE, IL-4, IL-5, IL-10, IL-13, and IFN-γ and eosinophil infiltration in nasal mucosa were significantly reduced due to treatment with onion extract. Hence, the topical administration of onion extract affects allergic symptoms through reducing Th1 and Th2 responses in allergic disorders [119].

In two other studies, *A. cepa* significantly inhibited IgE-induced histamine and beta-hexosaminidase release from RBL-2H3 cells [120]. In addition, the effects of onion peel hot water extract on cell viability, nitric oxide (NO), proinflammatory cytokines such as IL-6, TNF-α, and IL-1β, murine macrophage cell line, and RAW 264.7 from Balb/c mice with croton oil-induced mouse ear edema were examined. The level of NO, IL-6, TNF-α, and IL-1β production by OPHWE was decreased dose dependently compared with the lipopolysaccharide (LPS) group, indicating the anti-inflammatory and immunomodulatory activities of onion peel hot water extract. These results suggested that onion could be regarded as a candidate for the treatment of inflammatory and immune-dysregulatory disorders [121].

Dorsch et al. showed antiasthmatic effect of the *A. cepa* extract caused by improvement of leukotriene and thromboxane biosynthesis as well as histamine release. The efficacy of *A. cepa* in allergic diseases was indicated by improvement of leukotriene and thromboxane biosynthesis as well as histamine release [122, 123].The levels of TNF-α and IL-12 and phagocytosis in cultured peritoneal cells from mice were increased due to oral administration of the mucus of bunching onion. In addition, production of IFN-γ from spleen cells and natural killer (NK) activity were augmented in the treated groups, indicating increased natural immunity by oral onion.

The effect of a herbal fraction (ALC-02) from *A. cepa* on type I allergic reactions was shown to be mediated by inhibiting histamine release and reduction of intracellular calcium levels, as well as preventing systemic anaphylaxis and decreasing histamine levels and lipid peroxidation in compound 48/80-induced rat peritoneal mast cells. Carrageenan-induced rat paw edema, eosinophil peroxidase activity, and protein content in the BALF of OVA-sensitized mice ALC-02 were also reduced in the treated group. These findings showed the antiallergic property of *A. cepa* mediated by its potential antihistaminic, anti-inflammatory, antioxidant, and immunoregulatory activities [31].

In White Leghorn chickens, concentration-dependent inhibitory effects of garlic and onion extracts (0.8–409.6 μg/ml) on cell proliferation and IL-2 and INF-γ gene expression of stimulated lymphocytes were shown which support the immunomodulatory effects of the two plants [124]. The effect of aqueous garlic and onion extracts (150 and 400 mg/kg/day, orally) during the last 8 weeks of fructose feeding (for 14 weeks) in thirty-day-old male Wistar rats was studied. Garlic and onion treatment decreased oxidative stress, increased eNOS activity, and reduced vascular cell adhesion molecule-1 (VCAM-1) expression which provided new evidence on anti-inflammatory and immunomodulatory effect of garlic and onion [125].

Atypical prostatic hyperplasia (APH) induced by subcutaneous (s.c.) injection of testosterone (0.5 mg/rat/day) and through smearing citral on the skin once every 3 days for 30 days was treated with onion suspension (75, 150, and 300 mg/kg/day; oral) and palmetto (100 mg/kg) was used as a positive control for 30 days. The results showed decreased IL-6, IL-8, and TNF-α which was dose dependent. These findings showed potential anti-inflammatory and immunomodulatory effects of the extract of onion as indicated by protective effects against APH induction in rats [48].

Application of *A. cepa* on the nasal cavity of BALB/c mice with allergic rhinitis revealed remarkable decreases in IgE and inflammatory cytokines IL-4, IL-5, IL-10, and IL-13. In addition, eosinophil infiltration into nasal turbinate

mucosa was also considerably decreased [119]. In addition, it has been shown that *A. cepa* decreased vascular permeability leading to reduction of BALF protein exudation [31].

9.1.2. Clinical Evidence. Several clinical studies demonstrated the effect of *A. cepa* on allergic and immunologic disorders. The effect of *A. cepa* supplementation (500 mg twice a day) on 419 cases with respiratory and allergic diseases showed a reduction in TNF-α and IL-6 [126].

The intranasal application of onion seed for 2 weeks in a cohort of 66 cases with allergic rhinitis reduced the nasal mucosal congestion, nasal itching, runny nose, sneezing attacks, turbinate hypertrophy, and mucosal pallor as well as IgE level and eosinophil count in nasal discharge during the first two weeks of treatment. Also, attenuation of the clinical symptoms of allergic rhinitis by stabilizing mast cell membranes was seen [127].

However, induction of allergic reaction to onion was also indicated in a number of studies. An episode of anaphylaxis following cooked onion ingestion was reported which was confirmed by skin test, and immuno CAP confirmed the IgE-dependent response to onion in this patient. In addition, only B cells were proliferated in response to onion extract. Therefore, cooked onion can induce severe allergic reactions, indicating the presence of thermostable components [2].

The effect of onion extracts on 2508 subjects with food intake-related symptoms and food hypersensitivity identified by the skin test, positive specific IgE, or provocation in 924 cases was examined. In 27 of these cases, food intake-related symptoms occurred following onion intake. Also, according to immunodetection results, an association between the symptoms and a specific lipid transfer protein (LTP) to the bulbs of onion was shown [128]. Therefore, allergic hypersensitivity to onions should not be underestimated and should be included in the diagnostic food allergy protocol [128].

9.2. Effects of the Plant's Constituents

9.2.1. Experimental Evidence. Flavonoids in onions, such as Qt and kaempferol, showed various biological roles in health maintenance such as antiviral, antimicrobial, anti-inflammatory, anticancer, and immuno-modulatory activities [129, 130]. Various effects were reported for Qt such as stimulation of the immune system, antiviral activity, inhibition of histamine release, and suppression of proinflammatory cytokines and leukotrienes (e.g., IL-4). Qt also improved the Th1/Th2 balance, restrained antigen-specific IgE antibody formation, and suppressed lipoxygenase, eosinophil, and peroxidase activities and inflammatory mediator levels. Therefore, Qt with anti-inflammatory and immune-modulating properties could be regarded as a candidate in the treatment of asthma, allergic rhinitis, and restricted peanut-induced anaphylactic reactions [131].

A number of studies indicated that Qt treatment decreased LPS-induced IL-8 production in lung A54 cells and mRNA levels of TNF-α and IL-1α in glial cells, production of cyclooxygenase (COX), lipoxygenase (LOX), and FcεRI-mediated release of proinflammatory cytokines, tryptase, and histamine from human mast cells [132–134].

Qt could be a useful supplement for the management of eosinophil-mediated diseases, such as allergic rhinitis and asthma. Treatment with Qt (5.0, 7.5, 10.0, 15.0, 17.0, and 20.0 mg/kg, once a day for 3 weeks, orally) for *Mesocestoides corti* infection in BALB/c mice, suppressed eosinophil activation with a minimum concentration of 5.0 μM but did not affect eosinophil growth or IgE hyperproduction [135].

Administration of isoquercitrin 15 mg/kg, Qt 10 mg/kg, or dexamethasone (1 mg/kg, s.c.) to BALB/c mice sensitized with OVA reduced eosinophil counts in the BALF, blood and lung parenchyma, neutrophil counts in the blood, and IL-5 levels in the lung homogenate (only in isoquercitrin-treated mice). In addition, Qt and isoquercitrin suppressed eosinophilic inflammation, suggesting their potential treating effect on allergic disorders [65].

Treatment with Qt (0.1–25 μM, orally) blocked the airway epithelial cell IL-8 and monocyte chemoattractant protein- (MCP-) 1 expression by attenuating the signaling through a PI-3 kinase/protein kinase B (Akt)/nuclear factor (NF)-κB pathway and inhibited chemokine expression. Also, Qt inhibited allergen sensitization, iMCP-1 expression, and airways hyperresponsiveness [136].

The inhibitory effects of Qt on different isotypes of immunoglobulins such as IgM, IgG, and IgA *in vitro* in mitogen-stimulated cells were also reported [137]. The effect of Qt and kaempferol on eicosanoid and nitric oxide-generating enzymes and its effect on the expression of proinflammatory genes were shown. Flavonoids in onions, such as Qt and kaempferol, also showed various antiviral, antimicrobial, anti-inflammatory, anticancer, and immunomodulatory activities [129, 138].

Kaempferol, the other compounds of onions (1–20 μmol/L), inhibited eosinophil adhesion in activated airway epithelium at dose more than 10 μmol/L in the TNFα-induced airway epithelium insult of six-week-old male BALB/c mice. Also, kaempferol reduced allergic and inflammatory airway diseases by NF-κB signaling pathway [139]. In addition, kaempferol treatments attenuated the Th2-driven allergic airway disease in an experimental model of asthma induced by OVA challenge by decreasing the production of IL-5 and IL-13 and ameliorating airway hyperresponsiveness induced by OVA challenge. Kaempferol also inhibited IgE-mediated release of proinflammatory mediators from human mast cells [140].

Kaempferol suppressed OVA challenge-elicited airway inflammation by its immunomodulatory properties through antagonizing NF-κB activation [86].

The inhibitory effect of kaempferol on LPS-induced epithelial eotaxin-1 expression and TNF-α-induced eosinophil-epithelial interaction was shown. Kaempferol also decreased eosinophil recruitment and accumulation in OVA-exposed mice.

Another flavonoid from onion, fisetin, inhibited IgE-mediated release of proinflammatory mediator and Th2-type cytokines from human mast cells and basophils [130]. S-allyl cysteine (SAC), a constituent of *A. cepa* (ranging from 10 to 600 μmol/L), inhibited TNF-α-induced inflammation in splenocytes from asthmatic mice through inhibition of p38

Table 5: The effects of *A. cepa* and its constituents on allergic and immunologic disorders.

Study type	Plant preparations	Dose	Study models	Effects	Ref.
Exp	*A. cepa* extract	100 and 1,000 mg/kg/day	Antiallergic immune response	Improved Th1/Th2 balance; reduced proinflammatory cytokine levels	[131]
	A. cepa extract	150 and 300 mg/kg b.w,	Intranasal	Decreased cellular infiltration and eosinophil and lymphocyte count in the blood and BALF	[91]
	A. cepa extract	Onion 20 and 40 μl	Intranasal	Decreased mRNA levels of IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF-α	[119]
	A. cepa extract	10 mg/400 mL	Peritoneal cells	Decreased cytokine release, macrophage phagocytic activity, and NK cell activity	[152]
	A. cepa extract	20 μL	Lung eosinophilia infiltration	Reduced inflammatory cytokines and total cell counts in BALF and EPO in the lungs	[31]
	A. cepa extract	500 mg/kg	Lung and tissue	Decreased no, IL-6, TNF-α, IL-1β levels	[153]
	A. cepa extract	500 and 50 mg/kg/day	Pulmonary tissues	Low doses of onion was not toxic but high dose was toxic to rats	[154]
	A. cepa extract	250–600 mg	Allergic rhinitis	Reduced production of IL-4, IL-5, and IL-13	[30]
	Kaempferol	75 mg/kg	OVA-sensitized mice	Reduced Th2, Th17, IL-4, IL-17, and TGF-β but increased Treg cells, Th1/Th2 ratio, FOXP3, IFN-γ/IL-4 ratio, and IFN-γ gene expressions	[155]
	Kaempferol	20 μmol/l	OVA-sensitized mice	Decreased IL-1β-induced NF-κb p65 DNA-binding activity and nuclear c-Jun expression	[156]
	Kaempferol	—	OVA-sensitized rats	Reduced eosinophil count, IgE, IL-4, IL-5, IL-13, TNF-α, and IFN-γ levels	[157]
	Kaempferol	50 μM	OVA-sensitized rats	Inhibited LTB4 production without cytotoxicity	[158]
	SAC	10 or 20 mg/kg	OVA-sensitized mice	Reduced airway hyperresponsiveness, inflammatory cell counts, Th2-type cytokines in BALF, and serum OVA-specific IgE	[159]
	SAC	—	C57BL/6 mice	Alleviated clinical symptoms; improved TNF-α, IL-17, ADNP, MAP1LC3A, and MMP-9 levels	[160]
	SAC	—	MISD in mice	Inhibited TNF-α-induced activation of p38, JNK, and NF-$\kappa\beta$ pathways	[161]
	Kaempferol	0.46 g	OVA-sensitized guinea pigs	Decreased histamine and leukotrienes; increased PGE2	[162]
	Amentoflavone	—	Mouse ear and rat paw edema	Inhibited phospholipase A2 and cyclooxygenase pathway	[163]
	Quercetin	30 mg/kg	Bone marrow-derived mast cells	Decrease allergen-induced airway hyper responsiveness, Th2 responses in the lung, lung eosinophilia, and goblet cell metaplasia	[164]
	Kaempferol	10 or 20 μM	Knee osteoarthritic rats	Decreased TNF-α, IL-1β, and MDA levels	[165]
	Kaempferol	25, 50, 100, and 200 μm	Rat articular chondrocytes cultures	Reduced interleukin-1β-stimulated proinflammatory mediators by inhibiting the NF-$\kappa\beta$ pathway	[166]
	FFR + onion	150 and 400 mg/kg/d	Wistar rats	Decreased heart ENOS and VCAM-1	[125]
	Kaempferol	2 mg/kg/day or 10 mg/kg/day	Rat gingival tissues	Reduced INOS and TNF-α expression and nuclear p6; increased cytosolic p65; downregulated Erk, p38, JNK, and NIK/IKK expression	[167]
	Quercetin and kaempferol	10 and 20 μM	Rbl-2h3 intestinal cells	Inhibited the secretion of TNF-α and IL-4 in antigen-stimulated RBL-2H3 cells	[41]
	SAC	10 to 600 μmol/L	Splenocytes of asthmatic mice	Inhibited TNF-α-induced inflammation in HaCaT cells	[141]
	Thiosulfonates	100 mg/kg	Guinea pig model of asthma	Inhibited inflammatory cell influx by thiosulfinates	[95]
	Kaempferol	1–20 μmol/l	Asthmatic mice	Inhibited eosinophil adhesion to TNF-α-activated airway epithelium	[140]
	Kaempferol	0.22 μm	BALB/c mice	Inhibited IL-4-induced transcription factor STAT6 activation by specifically targeting Janus kinase 3	[139]
	Polygonum tinctorium Lour.	0.2, 2, or 20 mg/kg	OVA-induced mouse asthma	Reduced caspase-1 activity in nasal mucosa, IL-32, and IL-8	[168]
	Kaempferol	20 μm	BALB/c mice model of asthma	Suppressed LPS-induced IL-8 through the TLR4 activation; inhibited eotaxin-1 induction	[169]
	Kaempferol	—	LPS-stimulated THP-1 cells	Suppressed LPS-induced MDC, IP-10, IL-8, Th1, Th2, and neutrophil-related chemokines	[170]
	Kaempferol		Mouse model of asthma	Deceased inflammatory cell invasion, goblet cell hyperplasia, mucus secretion, regulated TNF-α, IL-4, IL-5, and IL-13	[171]
	Quercetin	40 μM	Allergic asthma	Inhibited rat tracheal contractility	[59]
	Quercetin	8 or 16 mg/kg	Allergic asthma BALB/c	Decreased allergic airway inflammation and hyperresponsiveness; improved Th1/Th2 balance via the suppression of GATA-3 and increase of T-bet expression	[32]
	Quercetin	25 mg/kg	Allergic asthma BALB/c	Inhibited nasal rubbing movements and sneezing	[172]
	Quercetin	20 mg/kg	Allergic asthma BALB/c	Inhibited nasal symptoms; increased TRX levels in nasal lavage fluids	[173]
Clin	*A. cepa* extract	Onion juice 52 g	Neuronal cell oxidative stress	Improved clinical symptoms; increased phagocytic and intracellular killing activities of PMNs and CD8 counts	[81]
	A. cepa extract	—	Allergic rhinitis	Reduced IgE and eosinophil count in nasal discharge; stabilized mast cell membranes	[127]
	A. cepa extract	—	Allergic disorders	Relieving perennial allergic rhinitis symptoms; increased phagocytic and intracellular killing activities of PMNs and CD8 counts	[174]
	A. cepa extract	50 g	Allergic induction	Severe allergic reactions induced by cooked onion	[175]
	A. cepa extract	—	Allergic hypersensitivity	Improved clinical symptoms	[128]

Ref.: references, Exp: experimental, Clin: clinical, IFN-γ: interferon- γ, TNF-α: tumor necrosis factor-α, BALF: bronchoalveolar lavage fluid, EPO: eosinophil peroxidase, BAL: bronchoalveolar lavage, MDA: malondialdehyde, NO_2: nitrite, NO_3: nitrate, SOD: superoxide dismutase, CAT: catalase, TC: total cholesterol, LDL-C: low-density lipoproteins, TG: triglycerides, HDL-C: high-density lipoproteins, PPAR γ: peroxisome proliferator-activated receptor γ, TSM: tracheal smooth muscle, i.p.: intraperitoneally, MMP-9: matrix metalloproteinase 9, MAP1LC3A: microtubule-associated proteins 1 A/1 B light chain 3A, ADNP: activity-dependent neuroprotector homeobox, ENOS: endothelial nitric oxide synthase, VCAM-1: vascular cell adhesion molecule-1, IL-17: interleukin 17, PGE2: prostaglandin E2, LTB4: leukotriene B4, THP-1: human monocytic cell line, OVA: ovalbumin, C57BL: C57 black 6, LPS: lipopolysaccharide, SAC: S-allyl cysteine, and MISD: multiple inflammatory skin diseases.

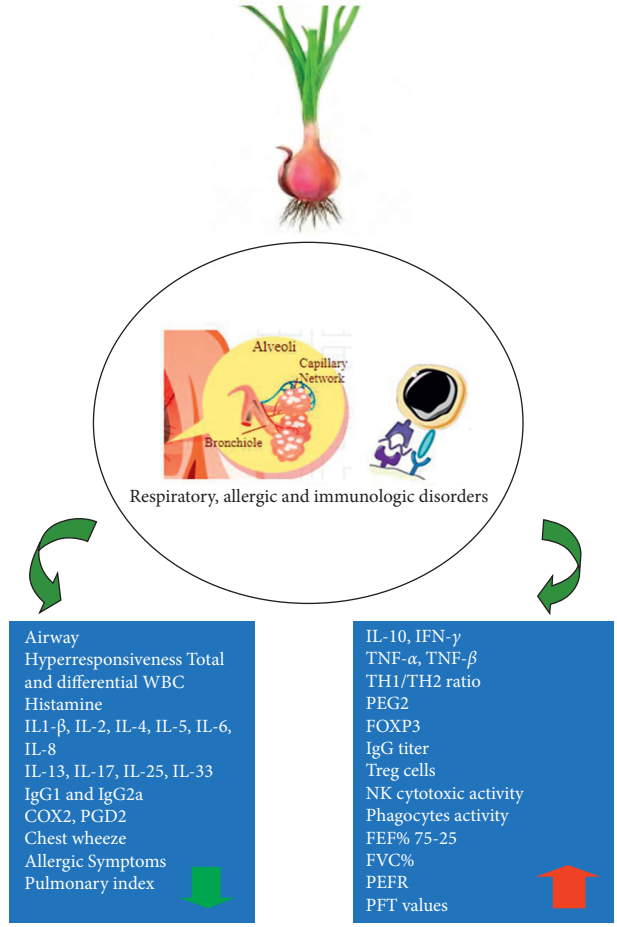

Figure 3: The possible molecular mechanisms of the preventive effects of *A. cepa* and its constituents on respiratory, allergic, and immunologic disorders.

and c-Jun N-terminal kinase (JNK) pathways and activation of extracellular signal-regulated kinase (ERK) [141].

9.2.2. Clinical Evidence. Treatment of patients aged 18–85 years with allergic rhinitis and upper respiratory tract infection (URTI) with Qt (500 and 1000 mg/day) decreased nasal mucosal congestion, nasal itching, runny nose, sneezing attacks, and mucosal pallor [6]. In a randomized, double-blind clinical trial with 58 patients, treatment with Qt capsules (five capsules twice a day for 12 weeks) relieved perennial symptoms of allergic rhinitis. However, Qt treatment did not reduce serum IgE, and therefore, the mode of action of Qt in reducing symptoms of allergic rhinitis could not be concluded in this study [16].

Consumption of 20 μM Qt and 20 μM kaempferol in allergic rhinitis patients decreased the release of IL-8 and MIP-3α and reduced nasal mucosal congestion, nasal itching, runny nose, sneezing attacks, turbinate hypertrophy, and mucosal pallor [12]. Kaempferol supplementation (72 mg/kg) in inhaled maintenance therapy reduced TNF-α and IL-6, the inflammatory biomarkers in male smokers [113].

In a nutritional-based clinical trial on healthy adults, cruciferous vegetable diets including kaempferol 270 mg/kg, broccoli 30–72 mg/kg, and radish 38 mg/kg were administered to the individuals for 14 days. The results showed reduction of IL-6 and IL-8 indicating the immuno-regulatory effects of these compounds [56, 86, 109, 142].

Occupational asthma induced by garlic dust was evaluated in 12 subjects employed in the garlic growing and processing industry. Five of the seven patients indicated garlic-specific IgE levels more than 0.7 kU/L as well as increased onion-specific IgE levels.

Clinical studies also showed reduction in laboratory markers for allergy including TNF-α and IL-6 as well as IgE and eosinophil count in nasal discharge and allergic symptoms including nasal mucosal congestion, nasal itching, runny nose, sneezing attacks, turbinate hypertrophy, and mucosal pallor in allergic rhinitis induced by onion, Qt, and kaempferol. Treatment of allergic rhinitis patients with kaempferol also reduced TNF-α, IL-6, IL-8, IL-1ß, and MIP-3α. The effects of *A. cepa* and its constituents on allergic disorders are summarized in Table 5, and mechanisms involved in such effects are presented in Figure 3. Experimental and clinical effects of *A. cepa* and its constituents on respiratory and allergic disorders are also shown in Figure 4.

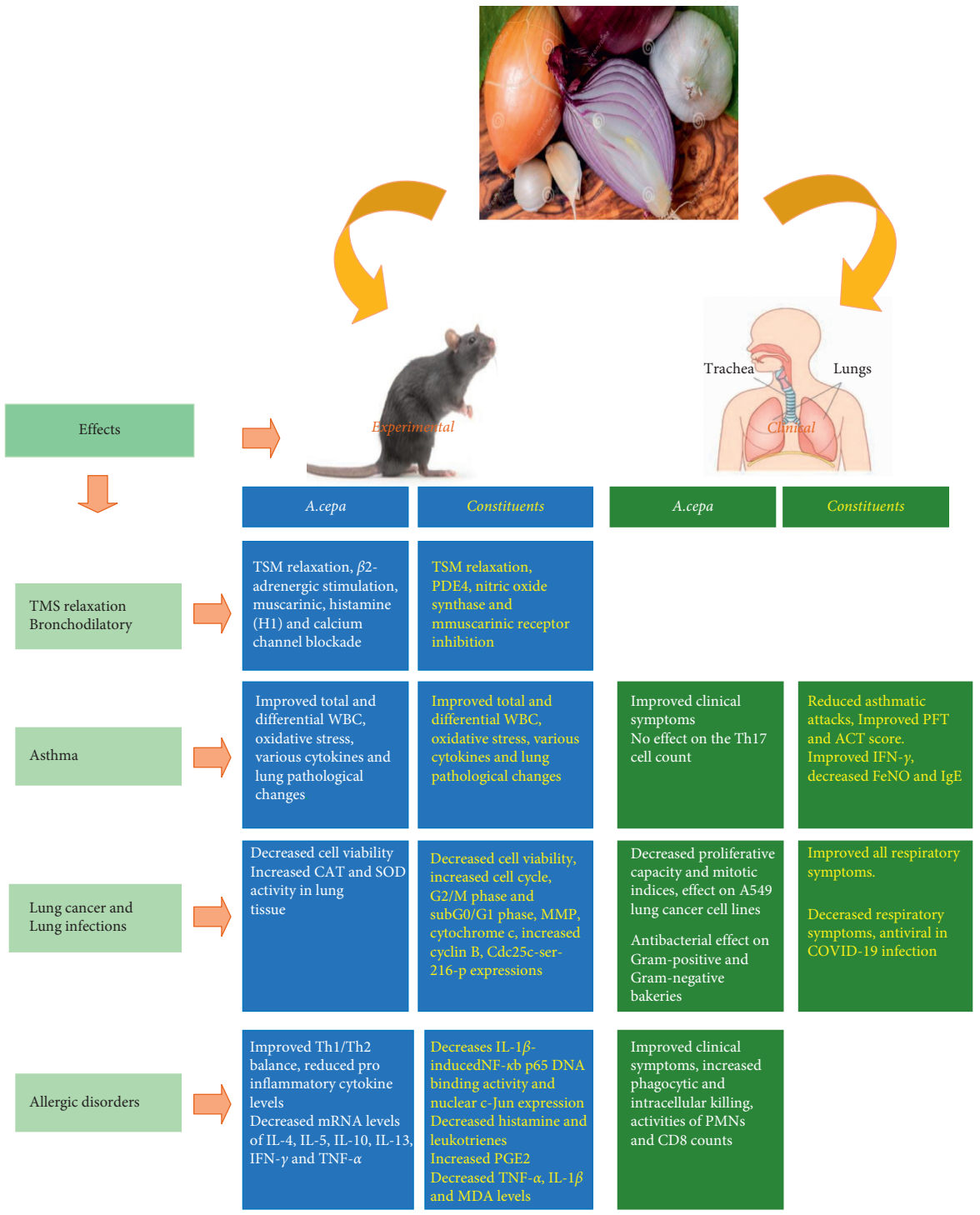

Figure 4: Experimental and clinical effects of *A. cepa* and its constituents on respiratory and allergic disorders.

10. Discussion

In this article, the potential effects of *A. cepa* and its constituents on various respiratory disorders based on experimental and clinical findings were reviewed.

Various experimental studies showed the relaxant effects of *A. cepa* and its constituents mainly Qt on TSM. The relaxant effects of the plant and Qt were possibly mediated by different mechanisms including β2-adrenoceptor stimulation, muscarinic and histamine H_1 receptor inhibition, calcium channel blocking, and phosphodiesterase enzyme-like mechanisms. These results suggest the possible bronchodilatory effects of the plant and Qt on obstructive respiratory diseases. However, further clinical studies are needed to examine this effect on asthma, chronic obstructive pulmonary diseases, or other obstructive pulmonary disorders.

Regarding the preventive effect of onion and its constituents on asthma, the AcE and the constituents of the plant mainly Qt decreased total and differential WBC in the blood and the BALF of animal models of asthma. Oxidant markers such as MDA were reduced, but antioxidants including CAT and SOD were increased in asthmatic animals by AcE and Qt. Serum and the BALF levels of PLA2, NF-κB, PGD_2, and GM-CSF were also decreased by AcE and Qt in the animal models of asthma. Treatment with the plant and Qt also decreased tracheal responsiveness and lung pathological changes in the sensitized animals. The level of IL-4 was decreased, but IFN-γ was increased and Th1/Th2 balance was improved in the animal models of asthma treated with AcE and Qt. Treatment with Qt also ameliorated asthma symptoms and protected asthma incidence in epidemiological studies. These results showed that *A. cepa* and its constituents could be considered possible preventive drugs for the treatment of asthma.

Treatment with the plant and Qt affects different cancer cell lines through modulating cell viability and other molecular mechanisms indicating their therapeutic effect on lung cancer. Clinical studies also support the effect of Qt on lung cancer.

The effect of extracts, essential oil, and the constituents of the plant, mainly Qt, on viral, microbial, parasitic, and fungal infections of the lung was shown. In experimental studies, beneficial effects of onion on lung infections caused by various viruses, bacteria, parasites, and fungi were reported. Treatment with Qt affects influenza virus infection and its lung manifestation. Clinical studies also showed the therapeutic effects of onion on symptoms of virus-infected flu. The effect of onion on the lung infected with *P. aeruginosa*, *S. aureus*, and *S. pneumonia* was also demonstrated. Treatment with Qt showed antiviral effects, and the effect of Qt on the treatment of COVID-19 patients was also indicated. The effect of Qt treatment on pulmonary tuberculosis was also demonstrated. Therefore, *A. cepa* and its constituents could be candidate drugs for treatment of various respiratory infections, especially viral infections and their lung manifestation mainly COVID-19.

Regarding the effect of onion and its constituents on allergic disorders, AcE treatment improved OVA-specific IgE, IL-4, IL-5, IL-10, IL-13, and IFN-γ levels in nasal mucosa and allergic symptoms in mouse models of allergic rhinitis; onion and its constituents inhibited cell proliferation, suppressed IL-2 and INF-γ gene expression in stimulated lymphocytes, and inhibited IgE-induced histamine and beta-hexosaminidase release from RBL-2H3 cells and production of IL-6, TNF-α, and IL-1β in murine macrophage cell lines. The plant also decreased VCAM-1 in fructose-fed rats and IL-6, IL-8, and TNF-α in APH condition. The plant and its constituents mainly Qt and kaempferol also decreased total and differential WBC and IL-4 in the blood and the BALF but increased IFN-γ, indicating enhanced Th1/Th2 balance both in the blood and the BALF of animal or cellular models of allergic disorders. The levels of IgM, IgG, and IgA in mitogen-stimulated cells and RANTES, MIP-1β, ECP, and MBP in the supernatants of cultured eosinophils from *M. corti*-infected mice were inhibited by Qt, and IL-5, IL-13, and IgE-mediated release of proinflammatory mediators was decreased by kaempferol. S-Allyl cysteine (SAC) also inhibited different cytokine gene expression in splenocytes of asthmatic mice and TNF-α-induced inflammation in HaCaT cells. Reduction in laboratory markers of allergy including TNF-α and IL-6, IL-8, IL-1ß MIP-3α, IgE, and eosinophil counts in nasal discharge and allergic symptoms including nasal mucosal congestion, nasal itching, runny nose, sneezing attacks, turbinate hypertrophy, and mucosal pallor in allergic rhinitis was decreased by the plant, Qt, and kaempferol in clinical studies. However, induction of allergic reaction to onion was indicated in a number of studies.

The current review article therefore indicates possible bronchodilatory and preventive effects of onion and Qt on asthma and other obstructive respiratory diseases. The effects of the plant and its constituents on lung cancer, lung infections, and allergic disorders were also reported both in experimental and clinical studies. However, before preparing drugs based on *A. cepa* and its constituents for clinical practice, further standard clinical trials are needed to be performed.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Endophytic Fungi from *Alstonia boonei* De Wild and *Greenwayodendron suaveolens* (Engl. and Diels) Verdc. subsp. *Suaveolens* Possess Inhibitory Activity against Pneumonia Causing Bacteria

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This study evaluated the antibacterial efficacy of methanolic extracts of isolated endophytic fungi from stem barks and leaves of *Alstonia boonei* De Wild and *Greenwayodendron suaveolens* (Engl. and Diels) Verdc. subsp. *Suaveolens* against *Klebsiella pneumoniae* ATCC 43816, *Haemophilus influenzae* ATCC 49247, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 35218, responsible for causing pneumonia. The endophytic fungi were isolated and characterized in the Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), and Czapek Dox Agar (CDA) media. The fungi and their methanolic extracts were tested for *in vitro* antibacterial potential by antagonistic assay for endophytic fungi against bacterial pathogens and microdilution method. The phytochemical screening of extracts was carried out according to the colorimetric and precipitation methods to reveal the presence of secondary metabolites. The results showed that 24 macroscopically and microscopically distinct endophytic fungi were isolated, identified, and stored. These endophytic fungi possessed antibacterial activity against the selected bacterial strains with inhibition zones ranging from 7.00 to 25.00 mm. The endophytic fungi GS_{15} and AB_{24} have presented the inhibitions zones of 20.33 mm and 25.00 mm, respectively, and these were better than the ones obtained for Levofloxacin®. The endophytes with inhibition zones greater than 10 mm were used for extraction of their secondary metabolites. The endophytic fungi extracts showed antibacterial activity with the minimum inhibitory concentrations (MICs) ranging from 6.25×10^{-4} to 2×10^{-2} g/L and the minimum bactericidal concentrations (MBCs) ranging from 2.5×10^{-3} to 2×10^{-2} g/L. The endophytic fungi GS_{15} extract was the most effective extract; it showed bactericidal effects on the tested bacterial strains. The phytochemical screening of the extracts revealed the presence of secondary metabolites classes, responsible for causing the obtained antibacterial activity. Thus, the endophytic fungi methanolic extracts from *A. boonei* and *G. suaveolens* have the potential to inhibit the growth of bacteria responsible for nosocomial pneumonia.

1. Introduction

Pneumonia is a lower respiratory tract infection caused by several infectious agents, including viruses, bacteria, and fungi [1]. However, pneumonia caused by bacterial infection has a rapidly progressive clinical course, which often becomes complicated by inflammation of the lungs, multilobular involvement, and lung abscesses [2, 3]. As the third deadliest infectious disease worldwide after tuberculosis and hepatitis B, it is also the leading cause of infectious deaths in children worldwide, but it is most prevalent in South Asia and sub-Saharan Africa. It accounted for 922,136 and 808,694 deaths in children below the age of five years in 2015 and 2017, respectively, representing up to 15% of all deaths of children below five years of age [4]. Hence, the pneumonia causing bacteria represents the leading cause of infant morbidity and mortality recorded in low-income countries [5] accounting for 16.5% deaths among children aged 0 to 59 months in Cameroon [6, 7]. In France, pneumonia is also responsible for 30% of deaths among adults over the age of 65 years. Appropriate antibiotic therapy involves Amoxicillin®/Clavulanic® acid and Levofloxacin® for patients with pneumonia [8]. However, this treatment is relatively expensive in low-income countries; meanwhile, the pneumonia causing Gram-negative bacteria is responsible for microbial resistance [9]. Therefore, this scenario necessitates exploration of the various possibilities for treatment of this disease [10]. Different therapeutic strategies have already been implemented for treatment of this disease; among which use of medicinal plants offers an inexhaustible source of drugs [11].

Alstonia boonei De Wild belongs to the Apocynaceae family. About 110 species of this genus grow alongside the American tropical region. Preparations using the leaves, seeds, stem barks, and roots of different plants from the Apocynaceae family have been largely used in traditional medicine and some plants of *Alstonia* genus have been widely used as a febrifuge to treat malaria and other skin problems. Alcoholic extracts of the stem barks of *A. boonei* (to a lesser degree of the leaf) showed a broad-spectrum activity against both Gram-negative and Gram-positive bacteria, as well as fungi [12].

According to Lissambou et al., *Greenwayodendron suaveolens* (Engl. and Diels) belonging to the Annonaceae family has long been used as food and herbal medicine in Central Africa, especially as powdered supplement [13]. The genus *Greenwayodendron* is traditionally used for its antimalarial, antimenorrhagic, and antidysenteric properties [14]. This genus is a rich source of biologically active secondary metabolites with antimicrobial [15, 16], analgesic [17], antimalarial [18], and anthelminthic [19] activities.

For centuries, plants have served as medicinal bioactive compounds source against many forms of disease. In contrast, during the last few years, microorganisms associated with plants rather than the plants themselves have been shown to offer products with high therapeutic potential [20]. All microorganisms (bacteria and fungi) that inhabit asymptomatically, at least for one period of their life cycle, the internal plant tissues beneath the epidermal cell layers may be considered as endophytes [21]. Their role is to improve plant's access to nutrients and produce special substances, mainly secondary metabolites and enzymes which are responsible for plant adaptation to abiotic stress [22]. During the long coevolution of endophytes and their host plants, endophytes have adapted themselves to their special microenvironments by genetic variation, including uptake of some plant DNA into their own genomes. This could have led to the ability of certain endophytes to biosynthesize some "phytochemicals" originally associated with the host plants [23]. Of the endophytic microorganisms, fungi have been isolated the most [24] as they have been proved to be the promising sources of biologically active products with antioxidant, immunosuppressive, antiproliferative, anti-inflammatory [23], and antibacterial activities [25]. Furthermore, considering the exponential evolution of antibiotic resistance in human pathogens in recent years, it has become inevitable to search for more efficacious antibiotics. Thus, the antimicrobial activities have been evaluated for a variety of metabolites biosynthesized by endophytic fungi [10, 24] and there is an increasing effort to characterize, identify, and evaluate the potential biological effects of endophytic fungal extracts isolated from medicinal plants [21]. However, the endophytes of *A. boonei* and *G. suaveolens* have not yet been characterized.

The present study evaluated the antibacterial potential of the methanolic extracts of endophytic fungi of stem barks and leaves of *Alstonia boonei* De Wild and stem barks of *Greenwayodendron suaveolens* (Engl. & Diels) Verdc. subsp. *Suaveolens* on the growth of several bacterial strains responsible for nosocomial pneumonia.

2. Material and Methods

2.1. Plant Materials. Healthy and mature plants without any visual disease symptom were carefully chosen for sampling. Leaves and stem barks of *A. boonei* were harvested from the botanical garden of the Institute of Medical Research and Medicinal Plants Studies, Yaoundé, Cameroon (Lat. 3° 51′ 39.298″ N; Long. 11° 30′ 19.192″ E) in the month of November 2018 and the stem barks of *G. suaveolens* were harvested in February 2019 at Mount Kala (Lat. 3° 52′ 28.515″ N; Long. 11° 27′ 9.335″ E) in Nkolbisson locality in Yaoundé, Cameroon. *A. boonei* and *G. suaveolens* samples were identified at the Cameroon National Herbarium under identification no. 43365/HNC and identification no. 45578/HNC, respectively.

2.2. Isolation and Cultivation of Endophytic Fungi. The plant samples were washed under running tap water, and sterilization of leaves surfaces was achieved by subsequently soaking them in a series of solutions as follows: sterile distilled water for 2 min, ethanol 70% for 2.5 min, sodium hypochlorite 2.4% for 4 min, and ethanol 70% for 1 min, and

finally they were rinsed with sterile distilled water for 3 times [26, 27]. They were dried in sterile absorbent paper. The last washing water was plated on Petri dishes containing Potato Dextrose Agar (PDA). The success of surface sterilization method was confirmed by the absence of any microbial growth on the culture media plated with the last washing water. The sterilized leaves were cut into 5 mm segments using a sterilized knife. Ten parts of each plant segments were placed in Petri dishes (9 cm) containing PDA supplemented by 0.5% of chloramphenicol and incubated at $28 \pm 2^{\circ}$C [26]. Regular observations were carried out from the second day onwards for a period of 3 to 4 weeks for fungal growth. The fungal growth from internal tissues was checked for purity and transferred to fresh culture slants and then stored at 4°C for further study [27].

2.3. Identification of Endophytic Fungi. Identification of endophytic fungi associated with leaves or stem barks segments of plants was done based on their morphological and taxonomic properties (macroscopically and microscopically) on PDA, Sabouraud Dextrose Agar (SDA), and Czapek Dox Agar (CDA) media at $28 \pm 2^{\circ}$C [28–34]. The macroscopic characteristics observed were color and surface colonies (granular, such as flour, mounting, slippery), texture, zonation, growth area, the lines of radial and concentric, reverse color, and exudate drops. The microscopic examinations of the vegetative thallus, fruiting bodies, and spores were carried out with methylene blue and Congo red reagents. The species identification was done according to the methods described earlier [35–38].

2.4. Extraction of Secondary Metabolites of Endophytic Fungi

2.4.1. Fermentation. Four endophytic fungi previously purified with inhibition zones higher than 10 mm on the tested bacteria were chosen for this purpose. Thus, some mycelial agar plugs from these endophytic fungi culture were cultivated into 500 mL culture flask containing 250 grams of sterile rice. The culture was incubated at still condition at room temperature under dark condition for 3 to 4 weeks [39].

2.4.2. Extraction of Endophytic Fungal Culture. After incubation period, biomass and culture media were extracted with methanol. The methanolic fraction was evaporated under reduced pressure by Heidolph brand rotary evaporator.

2.5. Assessment of Antibacterial Activity

2.5.1. Bacterial Strains. The antibacterial activities were tested using qualitative biological analysis in triplicate. The pathogenic bacteria used in this study were *Haemophilus influenza* ATCC 49247, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218, and *Klebsiella pneumoniae* ATCC 43816. These bacterial strains were provided by the Laboratory of Bacteriology of Yaoundé University Hospital Centre (Cameroon).

2.5.2. Antagonistic Assay for Endophytic Fungi against Bacterial Pathogens. Antagonistic activities of twenty-four endophytic fungi were tested for their antibacterial activity [40]. The test bacteria grown on liquid Mueller-Hinton medium for 24 h; the concentration was adjusted at 1×10^6 cells/mL [41]. The bacteria (100 μL) were inoculated on the Petri dishes containing solid Mueller-Hinton medium. Afterwards, the disks of agar (Ø 6 mm) of each endophytic fungus young strain (5 to 7 days) were inoculated and placed at equidistant. The plates remained incubated at $37 \pm 2^{\circ}$C for 24 h. Levofloxacin® (Sigma) (100 μg/mL in dimethyl sulfoxide (4%; v/v)) was employed as positive control. All the experiments were carried out using a completely randomized design (CRD), with three repetitions [42].

The presence of zone clearance on agar plates was used as an indicator for the antibacterial activity of endophytic fungi under investigation. The strains, which showed the maximum zone of clearance, were chosen for further study. The presence of these inhibition zones on agar plates was used as indicator of bioactive potential of endophytic fungi strains [43, 44].

2.5.3. Microdilution Assay of Endophytic Fungi Extracts

(1) Determination of Minimum Inhibitory Concentration (MIC). The geometric serial broth microdilution method was carried out according to the Microplate Alamar Blue Assay (MABA) described previously by the Clinical and Laboratory Standards Institute. A stock solution was prepared by diluting the respective sample in dimethyl sulfoxide (4%; v/v). Stock solution was then added to Mueller-Hinton broth to reach final samples concentrations ranging from 2×10^{-2} g/L to 1.95×10^{-5} g/L. Bacterial inocula (1.5×10^8 CFU/mL) were added to each dilution. Each 96-well microtiter plate was incubated at 37°C for 24 h. Positive control consisted of Levofloxacin® at 2.5×10^{-4} g/L, negative control contained no drugs, and blank contained neither inoculum nor drug. The concentration of dimethyl sulfoxide in the assay was kept at 5% to ensure that its effect on bacterial growth can be maintained as minimal. Upon incubation periods, 40 μL of 0.02% resazurin was added to individual wells and the plates were reincubated for additional 30 min and checked for color change. Change in resazurin color from blue to pink indicated reduction of the indicator due to bacterial growth. The MIC was defined as the lowest concentration of samples at which the microorganisms did not demonstrate growth [45].

(2) Determination of Bactericidal Effect. The Minimal Bactericidal Concentrations (MBC) of promising methanolic extracts of endophytic fungi were assessed by subculturing MIC test microtiter plates on Mueller-Hinton medium. The MBC was considered as highest dilution or lowest concentration at which no growth occurred in the medium. All the experiments were done in triplicate [45].

The antibacterial effect was deemed bactericidal or bacteriostatic depending on the ratio of MBC to MIC. If MBC/MIC is lower than four, the antibacterial effect of endophytic fungi extracts is bactericidal and bacteriostatic when MBC/MIC was higher than four [46].

2.6. Phytochemical Screening of Endophytic Fungi Extracts. Preliminary phytochemical screening of secondary metabolites of methanolic extracts of endophytic fungi was carried out according to methods described by Harborne and Evans [47, 48].

2.7. Statistical Analysis. All experiments for antagonistic assays were conducted in triplicate and values of inhibition zones expressed in mm as the mean ± SD. Variations in means were analyzed using one-way analysis of variance (ANOVA) and means were statistically significant if $p < 0.05$.

3. Results

3.1. Isolation and Identification of Endophytic Fungi. A total of 83 endophytic fungi were isolated from *A. boonei* and *G. suaveolens* and grouped into 24 fungi isolates. It was found that 16 fungi isolates were identified, belonging to four different genera: *Aspergillus* (12.5%), *Fusarium* (37.5%), *Neoscytalidium* (8.33%), and *Acremonium* (8.33%). These genera belong to Deuteromycota division, more precisely to the Hyphomycetes class. They were divided into hyaline hyphomycetes (Moniliaceae order) with *Aspergillus* spp., *Fusarium* spp., *Acremonium* spp., and dark hyphomycetes (Dematiaceae order) with the only genus *Neoscytalidium* spp. being identified. However, the remaining 08 endophytic fungi could not be identified due to their issues related infertility (Table 1).

Some morphological and microscopical characteristics of isolated endophytic fungi are represented in Figure 1.

Isolates of the genus *Aspergillus* appeared with bright colors, which made their identification easy. Three species of this genus were identified in this study. The mycelial colonies of the isolated species are either powdery with black color (*Aspergillus* sp. 1) and green in PDA and SDA media but orange-yellow in CDA medium (*Aspergillus* sp. 3) or downy with black color in PDA and white in CDA and SDA media (*Aspergillus* sp. 2). These isolates were also characterized by a septate thallus and an unbranched conidiophore with varying lengths and shapes, ending in a bulge or vesicle bearing phialides or sometimes separated by metules, which is characteristic of the genus called *Aspergillus* head.

The colonies of isolates of the *Fusarium* genus were downy or cottony with different colors ranging from pinkish white for *Fusarium* sp. 1 and 2 to brown in PDA for *Fusarium* sp. 3 with other colors such as pink or yellow for *Fusarium* spp. 4, 7, 8, and 9, especially in SDA and CDA media. Microscopy presented septate vegetative thallus with cluster or chain arrangement of microconidia, fusiform macroconidia, curved and quite pointed at the tips of the phialides, and the early presence of chlamydospores as seen on *Fusarium* sp. 2, justifying their belonging to the *Fusarium* genus. The colonies of isolates of the *Acremonium* genus were woolly in SDA and CDA and very poor in filaments or even hairless in PDA. Different colors were obtained like white and pink in SDA medium with a slow growth rate for *Acremonium* sp. 2 and a rapid growth rate for *Acremonium* sp. 1, as well as milky-white and white in PDA and CDA media. Microscopy presented a vegetative thallus made up of septate filaments, fine, cylindrical phialides at their tips, and cylindrical conidia grouped together in clusters. The colonies produced by the genus *Neoscytalidium* were milky-white fluffy in PDA and CDA media or woolly beige for *Neoscytalidium* sp. 2 and hairless brown for *Neoscytalidium* sp. 1 in SDA media. Microscopically, the hyphae are septate and hyaline; others are larger and strongly pigmented dissociating into arthroconidia. However, fungi isolates such as 17, 18, and 19 were unidentified as they did not produce sporing structures on PDA, SDA, and CDA media but presented dirty white cotton growth that darkened with age differently in each isolate. Microscopy presented septate and dark hyphae in fungi 17 and 18 differently from fungus 19, which presented septate and hyaline hyphae.

Table 1: Summary of endophytic fungi isolated from *Alstonia boonei* and *Greenwayodendron suaveolens*.

No.	Codes	Sources	Host plant	Genera names
01	GS_{15}	Stem bark	*G. suaveolens*	*Aspergillus* sp. 1
02	GS_{81}	Stem bark	*G. suaveolens*	*Aspergillus* sp. 2
03	AB_{58}	Stem bark	*A. boonei*	*Aspergillus* sp. 3
04	ab_{24}	Stem bark	*A. boonei*	*Fusarium* sp. 1
05	ab_{17}	Stem bark	*A. boonei*	*Fusarium* sp. 2
06	ab_{34}	Stem bark	*A. boonei*	*Fusarium* sp. 3
07	ab_{46}	Stem bark	*A. boonei*	*Fusarium* sp. 4
08	ab_{50}	Leave	*A. boonei*	*Fusarium* sp. 5
09	ab_{59}	Stem bark	*A. boonei*	*Fusarium* sp. 6
10	ab_{57}	Stem bark	*A. boonei*	*Fusarium* sp. 7
11	ab_{80}	Stem bark	*A. boonei*	*Fusarium* sp. 8
12	ab_{47}	Leave	*A. boonei*	*Fusarium* sp. 9
13	ab_{77}	Stem bark	*A. boonei*	*Acremonium* sp. 1
14	ab_{83}	Leave	*A. boonei*	*Acremonium* sp. 2
15	ab_{45}	Leave	*A. boonei*	*Neoscytalidium* sp. 1
16	ab_{38}	Stem bark	*A. boonei*	*Neoscytalidium* sp. 2
17	ab_{21}	Stem bark	*A. boonei*	*Not identified* 1
18	ab_{33}	Stem bark	*A. boonei*	*Not identified* 2
19	ab_{36}	Leave	*A. boonei*	*Not identified* 3
20	ab_{65}	Leave	*A. boonei*	*Not identified* 4
21	ab_{23}	Stem bark	*A. boonei*	*Not identified* 5
22	ab_{69}	Stem bark	*A. boonei*	*Not identified* 6
23	ab_{78}	Stem bark	*A. boonei*	*Not identified* 7
24	ab_{72}	Leave	*A. boonei*	*Not identified* 8

AB: *Alstonia boonei*; GS: *Greenwayodendron suaveolens*.

3.2. Antagonistic Potential of Endophytic Fungi. In this investigation, four endophytic fungi (GS_{15}, AB_{24}, AB_{38}, and AB_{83}) were selected for determination of inhibition parameters (MIC and MBC) after testing the 24 fungi isolates

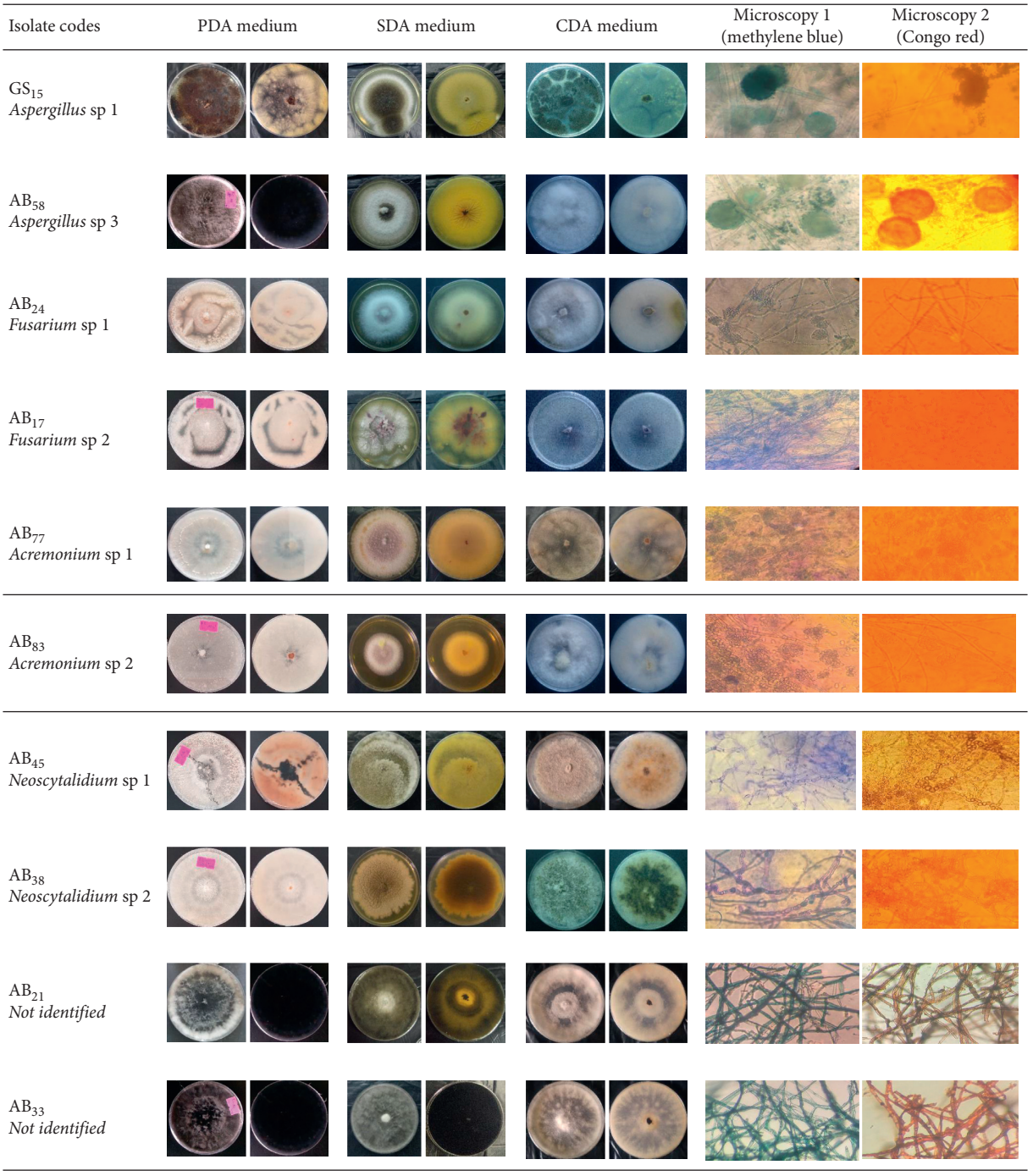

Figure 1: Morphology and microscopic characters of endophytic fungi isolated from *Alstonia boonei* and *Greenwayodendron suaveolens*.

from *A. boonei* and *G. suaveolens* on the growth of *H. influenza*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. These fungi isolates (GS_{15}, AB_{24}, AB_{38}, and AB_{83}) presented an antibacterial activity, with the inhibition zones ranging from 10.33 ± 0.57 mm against *E. coli* to 25.00 ± 1.00 mm against *H. influenza*. This was the most sensitive bacteria compared to those selected endophytic fungi with inhibition zone ranging from 20.33 ± 0.57 mm for AB_{24} to 25.00 ± 1.00 mm for GS_{15}; the data obtained was compared to Levofloxacin® with an inhibition zone of 19.05 ± 0.82 mm as in Table 2.

3.3. Antibacterial Activity of Endophytic Fungi Extracts. The antibacterial activities of the methanolic extracts of endophytic fungi from *A. boonei* and *G. suaveolens* are presented in Table 3. The methanolic extracts presented antibacterial activity against *H. influenza*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* with MIC values of 6.25×10^{-4}–2×10^{-2} g/L. The GS_{15} methanolic extract of endophytic fungi from *G. suaveolens* had the highest antibacterial activity with the MIC values of 6.25×10^{-4} g/L (*H. influenzae*), 2.5×10^{-3} g/L (*P. aeruginosa*), 2.5×10^{-3} g/L (*K. pneumoniae*), and 5×10^{-3} g/L (*E. coli*). The GS_{15}

Table 2: Inhibition zones produced by endophytic fungi isolates on the growth of *K. pneumoniae*, *H. influenzae*, *P. aeruginosa*, and *E. coli*.

Endophytic fungi isolates	Inhibition zones (mm)			
	H. influenzae	*K. pneumoniae*	*P. aeruginosa*	*E. coli*
GS_{15}	20.33 ± 1.52	19.00 ± 0.00	15.33 ± 0.57	10.33 ± 0.57
AB_{17}	22.66 ± 0.57	22.66 ± 0.57	7.00 ± 0.00	7.00 ± 0.00
AB_{21}	21.00 ± 1.00	17.66 ± 0.57	—	—
AB_{23}	24.66 ± 0.57	19.00 ± 0.00	—	8.33 ± 0.57
AB_{24}	25.00 ± 1.00	23.00 ± 0.00	20.00 ± 0.00	19.00 ± 0.00
AB_{33}	—	—	—	—
AB_{34}	17.33 ± 0.57	18.00 ± 0.00	8.33 ± 1.52	8.00 ± 0.00
AB_{36}	10.66 ± 0.57	—	7.00 ± 0.00	—
AB_{38}	20.33 ± 0.57	18.66 ± 0.57	19.00 ± 0.00	13.00 ± 1.00
AB_{45}	14.00 ± 1.00	15.33 ± 1.52	—	17.33 ± 0.57
AB_{46}	—	14.66 ± 1.52	8.00 ± 0.00	19.33 ± 0.57
AB_{47}	7.00 ± 0.00	15.33 ± 1.15	—	18.33 ± 1.15
AB_{50}	—	—	18.66 ± 1.52	—
AB_{57}	13.00 ± 1.00	—	11.00 ± 1.00	16.00 ± 0.00
AB_{58}	9.33 ± 0.57	—	—	—
AB_{59}	—	10.66 ± 1.15	—	18.66 ± 0.57
AB_{65}	—	12.00 ± 1.00	9.00 ± 0.00	17.00 ± 1.00
AB_{69}	—	9.66 ± 0.57	—	8.00 ± 1.00
AB_{72}	21.33 ± 1.15	—	—	7.00 ± 1.00
AB_{77}	18.00 ± 1.00	—	8.00 ± 1.00	10.33 ± 0.57
AB_{78}	8.33 ± 0.57	18.00 ± 0.57	7.00 ± 1.00	9.00 ± 1.00
AB_{80}	24.00 ± 0.00	21.00 ± 1.00	9.00 ± 1.00	7.00 ± 0.00
GS_{81}	16.00 ± 0.00	—	18.00 ± 1.00	13.00 ± 0.00
AB_{83}	23.33 ± 0.57	19.66 ± 1.15	15.00 ± 1.00	12.66 ± 0.00
Levofloxacin®	19.05 ± 0.82	19.51 ± 0.35	27.57 ± 6.74	20.37 ± 1.64

AB: *Alstonia boonei*; GS: *Greenwayodendron suaveolens*; —: not determined. Values are the means of three replicates ± SD.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for antibacterial activity of methanolic extracts of endophytic fungi from *A. boonei* and *G. suaveolens* against *K. pneumoniae*, *H. influenzae*, *P. aeruginosa*, and *E. coli*.

Samples	Bacterial strains	Inhibition parameters (g/L)			Antibacterial effects
		MIC	MBC	MBC/MIC	
GS_{15}	*H. influenzae*	6.25×10^{-4}	2.5×10^{-3}	4	Bactericidal
	P. aeruginosa	2.5×10^{-3}	5×10^{-3}	2	Bactericidal
	K. pneumoniae	2.5×10^{-3}	5×10^{-3}	2	Bactericidal
	E. coli	5×10^{-3}	2×10^{-2}	4	Bactericidal
AB_{24}	*H. influenzae*	2×10^{-2}	$>2\times10^{-2}$	—	—
	P. aeruginosa	2×10^{-2}	$>2\times10^{-2}$	—	—
	K. pneumoniae	2×10^{-2}	$>2\times10^{-2}$	—	—
	E. coli	$>2\times10^{-2}$	—	—	—
AB_{38}	*H. influenzae*	10^{-2}	2×10^{-2}	2	Bactericidal
	P. aeruginosa	10^{-2}	$>2\times10^{-2}$	—	—
	K. pneumoniae	10^{-2}	$>2\times10^{-2}$	—	—
	E. coli	2×10^{-2}	$>2\times10^{-2}$	—	—
AB_{83}	*H. influenzae*	2×10^{-2}	$>2\times10^{-2}$	—	—
	P. aeruginosa	2×10^{-2}	$>2\times10^{-2}$	—	—
	K. pneumoniae	2×10^{-2}	$>2\times10^{-2}$	—	—
	E. coli	2×10^{-2}	$>2\times10^{-2}$	—	—
Levofloxacin®	*H. influenzae*	78×10^{-7}	625×10^{-7}	8	Bacteriostatic
	P. aeruginosa	25×10^{-5}	25×10^{-5}	1	Bactericidal
	K. pneumoniae	$>25\times10^{-5}$	—	—	—
	E. coli	$>25\times10^{-5}$	—	—	—

AB: *Alstonia boonei*; GS: *Greenwayodendron suaveolens*; GS_{15}: methanolic extract of *Aspergillus* spp. 1; AB_{24}: methanolic extract of *Fusarium* spp. 1; AB_{38}: methanolic extract of *Neoscytalidium* spp. 2 and AB_{83}: methanolic extract of *Acremonium* spp. 2; —: not determined.

methanolic extract is the only extract that presented a bactericidal effect on the growth of the four bacteria strains studied. AB_{38} also presented a bactericidal effect on *H. influenzae* compared to the bacteriostatic effect obtained for Levofloxacin® on the same bacteria.

3.4. Phytochemical Screening. The extracts of the selected endophytic fungi (GS_{15}, AB_{24}, AB_{38}, and AB_{83}) have been used for several phytochemical tests to determine the presence of different types of bioactive compounds in them. The results presented in Table 4 indicated the presence of 10 secondary metabolites families in the methanolic extracts: alkaloids, phenols, polyphenols, tannins, saponins, flavonoids, anthocyanins, coumarins, terpenoids, and sterols. Unlike other classes of secondary metabolites that were ubiquitous to all four methanolic extracts, tannins were found exclusively in AB_{38} methanolic extract of *Neoscytalidium* sp. 2.

4. Discussion

4.1. Morphological and Microscopical Characterization. Isolation and identification of endophytic fungi have highlighted 4 genera, *G. suaveolens* (*Aspergillus*) and *A. boonei* (*Aspergillus*, *Acremonium*, *Neoscytalidium*, and *Fusarium*). The obtained results are similar to those of Abdel-Motaal et al. [49] who isolated endophytic fungi of *Neoscytalidium*, *Aspergillus*, *Acremonium*, and *Fusarium* genera from *Hyoscyamus muticus*, an Egyptian plant belonging to the Apocynaceae family same as *A. boonei*. In contrast, the study of Tolulope et al. [50] reported that the isolation of *A. boonei* endophytic fungi harvested in Nigeria contained three characterizing genera (*Aspergillus*, *Microphoma*, and *Trichoderma*). These results could therefore justify the hypothesis that endophytic fungi isolated from plants belonging to the same genus or family belong to the same genera. This is the case of *A. scholaris* and *Hyoscyamus muticus* according to the studies of Mahapatra and Banerjee and Abdel-Motaal et al., respectively, which indicated the presence of 19 genera with four similar genera (*Neoscytalidium*, *Aspergillus*, *Acremonium*, and *Fusarium*) in agreement with our study [49, 51]. We noticed that the colonizing endophytic fungi from *A. boonei* and *G. suaveolens* in our study have low genera diversities.

In addition, the genus *Fusarium* was the predominant genus isolated from *A. boonei* and *G. suaveolens* with a frequency of 37.5%. Indeed, it is one of the genera most associated with higher plants. *Fusarium* is among the most common fungi in terrestrial ecosystems. They are found in cultivated soils in temperate and tropical regions [37], and their predominance among the fungi isolated in this study corroborates with the study of Ilyas et al. [52] who explained that several fungi of the genus *Fusarium* were plant colonizers. In addition, *A. boonei* was harvested in tropical areas, where soils are favorable for the growth of species of this genus.

Table 4: Phytochemical screening of the methanolic extracts of endophytic fungi (GS_{15}, AB_{24}, AB_{38}, and AB_{83}).

Secondary metabolites	Endophytic fungi extracts			
	GS_{15}	AB_{24}	AB_{38}	AB_{83}
Alkaloids	+++	++	+	+
Phenols	−	+++	++	++
Polyphenols	++	+	+	+
Flavonoids	+	++	++	++
Tannins	−	−	+	−
Anthocyanins	−	+	+	+
Coumarins	++	+	−	++
Saponins	−	+	++	++
Terpenoids	+++	+	+	+
Steroids	+++	+	+	+

AB: *Alstonia boonei*; GS: *Greenwayodendron suaveolens*; GS_{15}: methanolic extract of *Aspergillus* sp. 1; AB_{24}: methanolic extract of *Fusarium* sp. 1; AB_{38}: methanolic extract of *Neoscytalidium* sp. 2 and AB_{83}: methanolic extract of *Acremonium* sp. 2.

4.2. Antibacterial Activity of Endophytic Fungi Extracts

4.2.1. Antagonistic Potential of Endophytic Fungi. The antagonistic potential of endophytic fungi (GS_{15}, AB_{24}, AB_{38}, and AB_{83}) allowed obtaining clearance zones on agar plates against *H. influenzae*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli* which are greater than 10.33 mm on the bacterial strains tested. The present inhibition justifies the antibacterial activity of these endophytic fungi strains on the bacteria responsible for pneumonia, which could be due to the presence of bioactive substances in the mycelial discs of these endophytic fungi [53]. However, other studies have shown that endophytic fungi isolated from plants could have antibacterial activity, so they would resist invasion and inhibit the bacteria inducing the diseases in humans through the production of inhibitory substances belonging to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids [24, 54, 55]. Our results corroborate with an earlier report in which the endophytic fungi studied belonged to *Aspergillus*, *Acremonium*, and *Trichosporon* genera isolated from *Artemisia absinthium*. These endophytic isolates showed antibacterial activity against *P. aeruginosa* ATCC27853 and *E. coli* ATCC25922 strains [56]. Although the tested strains responsible for pneumonia are naturally resistant due to their "Gram-negative" character, the obtained results show that the bioactive compounds secreted by these endophytic fungi would have the capacity to penetrate the external membranes surrounding the wall of Gram-negative bacteria made up of covering lipopolysaccharides by the phenomena of diffusion of lipophilic compounds. Another phenomenon, which could also explain the results obtained, is the diffusion of active substances on the agar, which depends on the nature of the diffusing substances excreted by the endophytic fungi, their concentrations, their solubility, and pH of the culture medium [57]. The high antibacterial activity revealed by endophytic fungi isolated from *A. boonei* and *G. suaveolens* is related to their ability to produce bioactive substances with medicinal properties [58].

4.2.2. Antibacterial Activity of Endophytic Fungi Extracts. The determination of the inhibition parameters (MIC and MBC) of methanolic extracts is shown by their MICs values of 6.25×10^{-4} to 2×10^{-2} g/L for *H. influenzae*, *P. aeruginosa*, *E. coli*, and *K. pneumonia* strains. The highest antibacterial activity was obtained with the methanolic extract of *Aspergillus* sp. 1 that presented a bactericidal effect on all tested strains. This could be linked to a synergistic or additive effect of the secondary metabolites present in the methanolic extract. In addition, the mean bactericidal effect of this extract on the strains tested is in line with data reported by Amadi and Adeniyi, as well as those reported by Indira et al. [59, 60] who demonstrated the antimicrobial activity of the genus *Aspergillus*. The reduction mechanisms of bacteria or fungi growth of the *Aspergillus* genus (*Aspergillus terreus*, *Aspergillus flavus*, and *Aspergillus fumigatus*) endophytes may possess higher concentration of alkaloids (fumiclavin A and B and, fumigaclavin B). In addition, most of these complex structures of fungi metabolites inhibit cell division and glucose transport [59, 61]. The data obtained in the present study were different from those of Kalhouche and Meziane [62] showing the MIC value of *Aspergillus* sp. 1 extract against *E. coli* to be 100 mg/mL. This difference may be because the *Aspergillus* strain used to inhibit the *E. coli* growth was not isolated from a medicinal plant. Hence, the production of secondary metabolites may not have been directed to any phytoprotective activity of plants. The methanolic extracts of AB_{24}, AB_{38}, and AB_{83} exhibited MICs ranging from 10^{-2} to 2×10^{-2} g/L. Indeed, the phytochemical screening of these extracts revealed the presence of phenols, flavonoids, and coumarins whose antibacterial effect could have been inhibited or neutralized by other compounds present in these extracts at higher concentrations [63]. These bioactive secondary metabolites would have the ability to penetrate the lipopolysaccharides of the wall of the Gram-negative bacteria tested and inhibit their growth by mechanisms of action that allowed them to inactivate bacterial enzymes, modify the structure of the active site, and induce intracellular accumulation of bioactive metabolites [64]. The bioactive compounds found in the methanolic extracts of endophytic fungi of *A. boonei* and *G. suaveolens* are responsible for the antibacterial activity and may have a synergistic action [65].

4.3. Phytochemical Screening of Endophytic Fungi Extracts. The phytochemical screening of the methanolic extracts of endophytic fungi isolated from *A. boonei* and *G. suaveolens* has shown that all the extracts contain alkaloids, polyphenols, flavonoids, terpenes, and sterols. The presence of these secondary metabolites in the endophytic fungi could justify their antibacterial effect. Previous studies have shown that phytochemical compositions of *A. boonei* and *G. suaveolens* stem barks extracts were similar to those in the present study with the presence of alkaloids, phenols, polyphenols, flavonoids, anthocyanins, saponins, terpenoids, and sterols [66, 67].

These studies permitted highlighting the phytomolecular mimicry of endophytic fungi extract from *A. boonei* and *G. suaveolens*. It appears that the phytochemical compositions of the endophytic fungi methanolic extracts of these plants would be similar to those of plant extracts [66, 67]. Therefore, to verify this hypothesis, it would be necessary in a further study to make additional quantitative and qualitative analysis, especially molecular and genetic levels between endophytic fungi and the host plant.

5. Conclusion

In this study, the endophytic fungi from *A. boonei* and *G. suaveolens* exhibited antibacterial activity against some of the human pathogenic bacteria responsible for nosocomial pneumonia. This activity was greater in the methanolic extract of GS_{15} endophytic fungal isolated from *G. suaveolens*, a plant that has never been listed for its antipneumonic potential. The present study helped to valorize the use of endophytic fungi from *A. boonei* and *G. suaveolens* in drug discovery process. However, a molecular identification of isolated endophytic fungi from these plants is necessary for further investigations.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

P. C. E. D. participated in the isolation and characterization of endophytic fungi and revised the manuscript. P. H. D. B. initiated the project, participated in laboratory works analyzing the presence of secondary metabolites, isolation, and characterization of endophytic fungi and biological activities, and revised the manuscript. C. W. K. participated in the characterization of endophytic fungi and revision of the manuscript. E. N. T. participated in the isolation and characterization of endophytic fungi. E. D. F. N. M. participated in the characterization of endophytic fungi and revised the manuscript. J. S. F. M. participated in the characterization of endophytic fungi and revision of the manuscript. G. A. A. revised the manuscript. M. A. N. initiated the project, guided the research work, and revised the manuscript. All authors read and approved the final version of the manuscript.

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Research Article

Mangiferin Inhibits Human Lung Adenocarcinoma by Suppressing MiR-27b and MiR-92a

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Lung adenocarcinoma (LUAD) is one of the most prevalent malignancies. However, its mechanism and therapeutic strategy remain to be clarified. Mangiferin is a flavonoid derived from the leaves of mango trees of the lacquer family that has many pharmacological and physiological effects. This research aimed to elucidate the biological effect of mangiferin in LUAD cell lines and clarify the in vitro mechanism of mangiferin. Mangiferin was shown to significantly restrain the proliferation of LUAD cells (A549, H1299, and H2030 cells) in a dose- and time-dependent manner. Furthermore, mangiferin was capable of stimulating apoptosis, and more cells were blocked in G1 and S phase in the mangiferin-treated cells than in those not treated with mangiferin. Microarrays and micro-RNA sequencing data suggested that there is a higher level of miR-27b and miR-92a in LUAD tissues than in non-LUAD tissues. Additional experiments indicated that mangiferin may be related to the downregulated levels of miR-92a and miR-27b. In conclusion, mangiferin likely regulates proliferation and apoptosis in LUAD cells by reducing the expression levels of miR-92a and miR-27b.

1. Introduction

Lung cancer (LC), a highly heterogeneous malignancy, contributes to nearly a quarter of cancer-related deaths globally, with almost 45% of cases being lung adenocarcinoma (LUAD) [1, 2]. Over 60% of LC patients are diagnosed at a locally metastatic or advanced stage owing to the lack of techniques and recognizable symptoms for early detection, and conventional surgery may not be a useful option for these patients. Although great progress in therapeutic strategies has improved the prognosis for some LUAD patients, the overall survival within 5 years is still less than 20% [3]. Thus, further investigation into novel agents for LUAD and their mechanisms is urgently needed.

Polyphenols are a kind of chemical substances abundant in plants, which play important roles in plant growth and metabolism. More and more evidence show that polyphenols have potential health properties on human body, such as antioxidant, antiallergic, and anti-inflammatory [4]. To our knowledge, many kinds of polyphenols can resist the

occurrence and development of tumor. For example, curcumin inhibits the proliferation and migration of tumor cells and promotes apoptosis [5]. Lingonberry and bilberry have been reported to have effects on gastrointestinal cancer [6]. Looking for new anticancer polyphenols may help to improve the current therapy.

Mangiferin is derived from the leaves, stems, peels, and roots of *Mangifera indica* and other herbaceous plants [7] (Figure 1). Studies have described a variety of biological effects of mangiferin on the human body, including antitumor, antioxidant, antibacterial, and antiviral effects [8–11]. One in vitro experiment showed that mangiferin exerts an influence on cell cycle arrest and induces apoptosis in A549 cells [12]. In addition, several studies have suggested that mangiferin plays a reverse or inhibitory role in lung carcinogenesis [13, 14]. However, the precise molecular mechanism remains unknown.

Micro-RNAs (miRNAs) are not only important regulators of cell proliferation, differentiation, and apoptosis but also closely related to cell phenotype and human diseases [15]. Emerging evidence has indicated that upregulated miR-27b-5p (miR-27b) and miR-92a-1-3p (miR-92a) levels contribute to various types of cancers [16–19]. The overexpression of miR-92a has been shown to promote cellular activity and suppress the sensitivity of lung cancer cells to gefitinib [20–23]. MiR-27b has also been confirmed to be consistently upregulated in dysplastic tissues compared with normal tissues during gastric carcinoma development [24].

Therefore, it is hypothesized that mangiferin might perform its antitumor role by targeting miR-92a and miR-27b. In this paper, we aimed to confirm the biological function of mangiferin in LUAD cells (A549, H1299, and H2030 cells) and detect genetic alterations affecting miR-27b and miR-92a under treatment with mangiferin. Furthermore, an in silico analysis was performed with common targets of miR-27b and miR-92a to explore the underlying mechanism of mangiferin in LUAD.

2. Materials and Methods

2.1. Reagents. Mangiferin ($C_{19}H_{18}O_{11}$) was obtained from the Central Laboratory of the Institute of Clinical Medicine of Guangxi Nationalities Hospital. Mangiferin is a light-yellow crystal and is slightly soluble in water, ethanol, and methanol; soluble in hot dilute methanol and ethanol; and insoluble in nonpolar solvents. It was prepared at 100 mg/mL with 2% $NaHCO_3$ and packaged and frozen at 20°C. Fetal bovine serum (FBS) was purchased from HyClone (Thermo Fisher, Logan, Utah, USA).

2.2. Cell Lines for In Vitro Experiments. The H2030, H1299, and A549 (human LUAD) cell lines, obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI 1640 medium (Welgene, Gyeongsan, South Korea) and routinely supplemented with 10% (v/v) FBS in a 37°C incubator with 95% O_2 and 5% CO_2. The experimental groups were treated with different concentrations of mangiferin which dissolved in 2% $NaHCO_3$. And, the control group was treated with the same amount of 2% $NaHCO_3$.

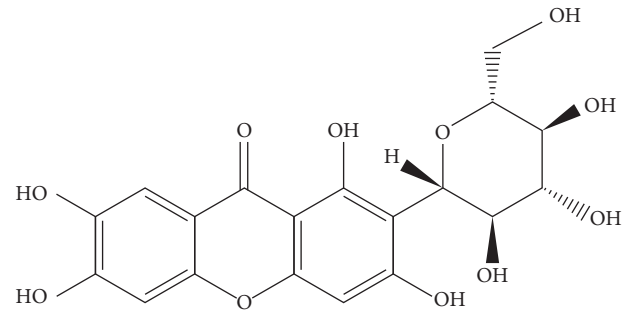


Figure 1: The chemical structure depiction of mangiferin ($C_{19}H_{18}O_{11}$).

2.3. Cell Proliferation Detection for LUAD Cell Lines. LUAD cells were seeded into 96-well culture plates, and cell proliferation was assessed by Cell Counting Kit-8 (CCK-8) assays at 12, 24, 36, 48, and 72 hours after adding 10 μL of different concentrations of mangiferin (12.5, 25, 50, and 100 mg/mL). LUAD cells were incubated for 1 hour, and then a microplate reader was used to read and analyze the sample at 450 nm (OD 450). Each experiment was performed according to the manufacturer's instructions and repeated three times.

2.4. Cell Cycle and Apoptosis Analyses. Collected cell lines were processed with prechilled PBS and then fixed with prechilled 70% ethanol at 4°C overnight. RNase A (50 mg/mL) was added to the cell suspension at 37°C for a 30-min incubation. The cell lines were washed with PBS and further processed with 30-min staining with propidium iodide (PI, 25 mg/mL) at 37°C. The characteristics of the cell cycle were detected using a FACS Calibur flow cytometer according to the manufacturer's instructions and further analyzed by ModFit software. The comparison rate (%) was computed as the proportion of cells in the G1/S/G2 phase.

Cell apoptosis was detected by an Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA). The cells were separated into four groups in each experiment: single-stained PI group, single-stained Annexin V group, PI and Annexin V double-stained group, and non-stained group. After a 15-minute incubation in Annexin V-FITC and PI, cell apoptosis was observed using a FACS Calibur flow cytometer. Each experiment was repeated three times and performed according to the manufacturer's instructions.

2.5. Quantitative Real-Time PCR (qRT-PCR). Human LUAD cells (A549, H1299 and H2030 cells) were treated with different concentrations of mangiferin (25, 50, and 100 μM) for 48 hours and further cultured for 24 hours. Then, qRT-PCR was performed to detect miR-27b and miR-92a expression levels in the cells. Total RNA was isolated from cells

with TRIzol reagent (Invitrogen, Braunschweig, Germany), and isolated RNA was further converted into cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Next, the relative expression of miR-27b and miR-92a in cells was assessed by ABI 7300 HT software, and U6 was used as an endogenous control to normalize miRNA expression. The sequences used for analysis are as follows: (miR-27b) forward 5'-AGAGCTTAGCTGATTGGTGAAC-3' and reverse 5'-GTTCACCAATCAGCTAAGCTCT-3'; and (miR-92a) forward 5'-AGGTTGGGATCGGTTG-CAATGCT-3' and reverse 5'-AGCATTGCAACCGATCC-CAACCT-3'.

2.6. Integrated Analysis of the Functional Mechanisms of MiR-27b and MiR-92a. The expression of miR-92a and miR-27b in LUAD samples was analyzed by integrating microarray data from the Gene Expression Omnibus (GEO) and microRNA-seq data from The Cancer Genome Atlas (TCGA). The miRWalk 2.0 database was used to predict the genes targeted by miR-27b and miR-92a [25]. The common target genes were subjected to further enrichment analysis via the DAVID database [26], and the potential regulatory network between these genes was analyzed in the STRING database (https://string-db.org/) [27].

2.7. Statistical Analysis. Data were processed and analyzed in SPSS 23.0 software. The expression levels of miR-27b and miR-92a are presented as the means ± SDs, and Student's *t*-test and one-way analysis of variance (ANOVA) were applied when comparing the statistical significance between different groups. The results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Mangiferin Inhibits the Proliferation of LUAD Cells. To study the role of mangiferin (10, 20, and 40 μM) on the proliferation of LUAD cell lines, the activity of LUAD cells was assessed by CCK-8 assay. Mangiferin showed inhibitory effects on A549, H1299, and H2030 cells, and the inhibition rate increased with increasing concentration. In addition, as the exposure time increased, the cell inhibition rate also showed an upward trend, which suggests that the inhibitory effect of mangiferin on LUAD cells is dose- and time-dependent (Figure 2).

3.2. Cell Cycle Distribution. After treatment with mangiferin, cell cycle analysis was performed to explore the cell cycle features of LUAD cells. Statistical analysis of G0/G1, S, and G2/M phase cell numbers showed that more cells were blocked in the G1 phase after mangiferin treatment than without mangiferin treatment, thus suggesting that mangiferin may induce A549, H1299, and H2030 cells to undergo apoptosis in a time-dependent manner (Figure 3). Moreover, S-phase arrest was discovered in the mangiferin-treated cells (Figure 4). The number of apoptotic LUAD cells (H1299, A549, and H2030 cells) after treatment with different concentrations of mangiferin (0, 12.5, and 100 mg/mL) varied with the number of apoptotic cells increasing with increasing concentration (Table 1).

3.3. Upregulation of MiR-27b and MiR-92a in LUAD Tissues. To explore whether miR-92a and miR-27b are differentially expressed in LUAD tissue relative to normal tissue, the GSE36681 dataset from the GEO database was downloaded for analysis [28]. The dataset contains 206 samples, of which 47 pairs of paraffin-embedded tissue and 56 pairs of fresh-frozen tissue were used. As shown in the figure, the expression levels of miR-27b and miR-92a in fresh-frozen (FF) LUAD tissues were significantly higher than those in noncancerous samples. However, consistent results were not observed in the subset of formalin-fixed, paraffin-embedded (FFPE) samples ($P > 0.05$). In addition, miRNA expression data from 495 samples (450 LUAD, 45 normal tissues) from TCGA confirmed that miR-27b and miR-92a showed higher levels in LUAD tissues than in non-LUAD adjacent tissues (Figure 4).

3.4. Downregulation of MiR-27b and MiR-92a after Mangiferin Treatment. Analysis of TCGA and GEO LUAD expression data showed that miR-27b and miR-92a showed higher levels in LUAD samples than in normal lung tissue (Figure 5). To investigate the biological effect of mangiferin on the miR-27b and miR-92a levels, cells (H1299 and H2030 cells) were treated with mangiferin (25 μM) for 24 hours, and then the levels of miR-27b and miR-92a were detected. The results suggested that miR-27b and miR-92a levels were clearly decreased in H1299 and H2030 cells after 24 hours of mangiferin treatment (Figure 6).

3.5. Functional Enrichment of MiR-27b and MiR-92a Target Genes and Analysis of Regulatory Networks. Potential target genes that appeared in 5 or more databases were included, from which we obtained 1196 targets of miR-27b-5p and 1589 genes targeted by miR-92a-1-5p. Finally, a total of 426 common genes were analyzed for enrichment. Gene Ontology (GO) analysis is used to determine the functions of genes at three levels: cellular component (CC), molecular function (MF), and biological process (BP) levels. The results indicated that the common target genes were mainly involved in the regulation of the RNA transcription process, and their main molecular functions included the regulation of transcriptional activity and the binding of transcription factors. Their positions in cells were mainly within the membrane fraction, insoluble fraction, and endoplasmic reticulum (Figure 7).

4. Discussion

This study aimed to investigate the repressive role of mangiferin and its potent mechanism in LUAD cells with the hope of identifying a novel therapeutic for LUAD patients. The present paper confirmed that mangiferin induces effects by analyzing three subsets of LUAD cells, and further

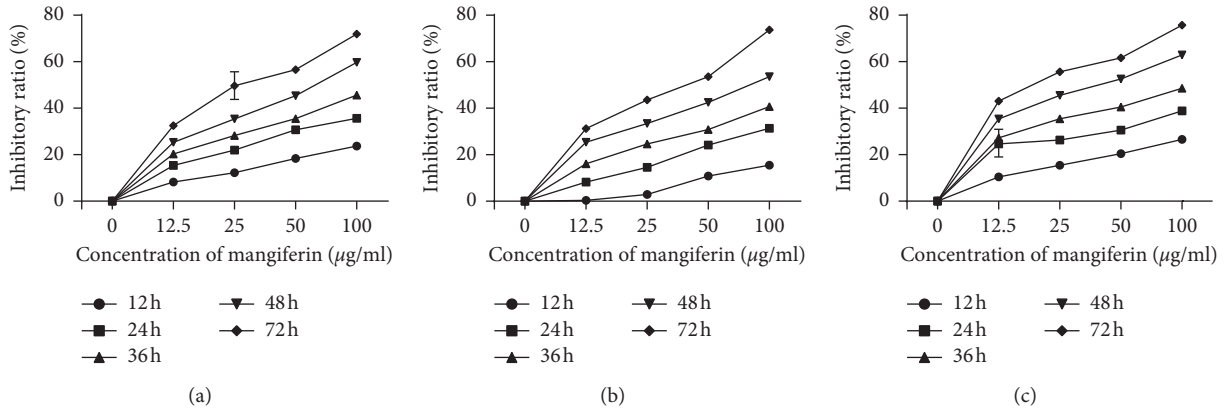


Figure 2: Mangiferin suppresses the proliferation of lung adenocarcinoma cells. (a) A549 cells. (b) H1299 cells. (c) H2030 cells.

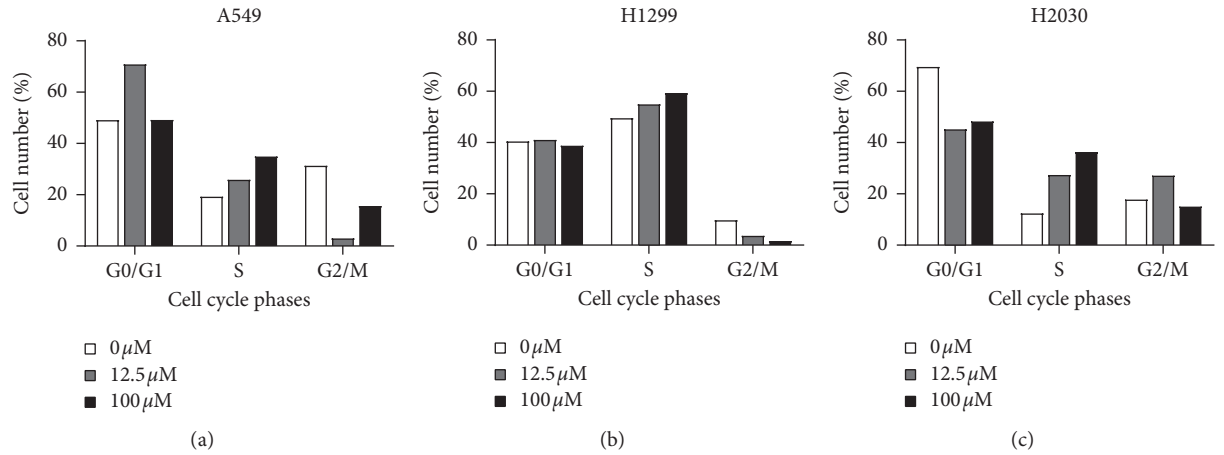


Figure 3: Cell cycle analysis was performed in A549 (a), H1299 (b), and H2030 (c) cells treated with mangiferin.

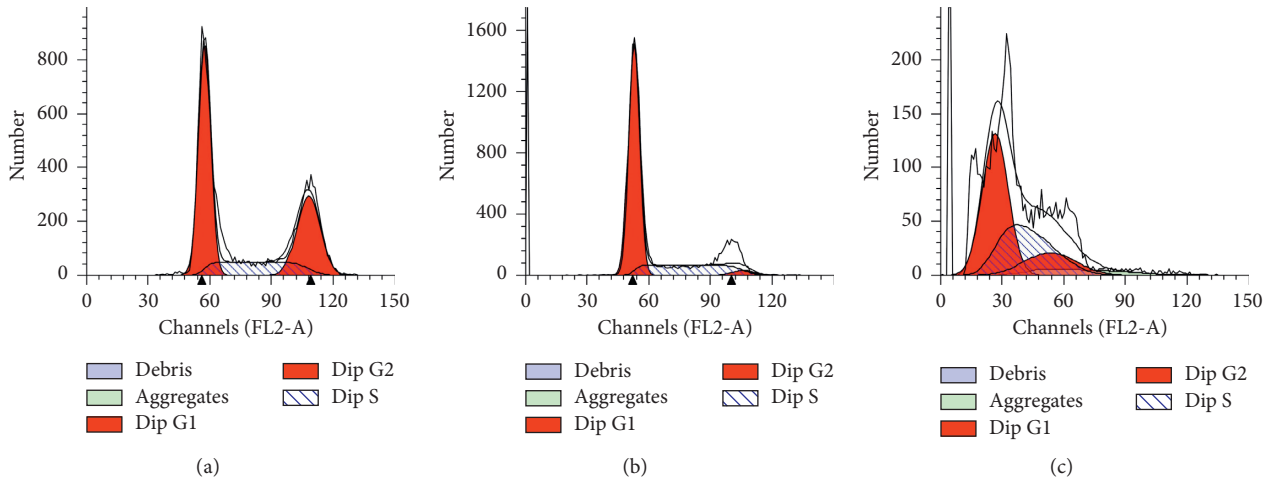


Figure 4: Continued.

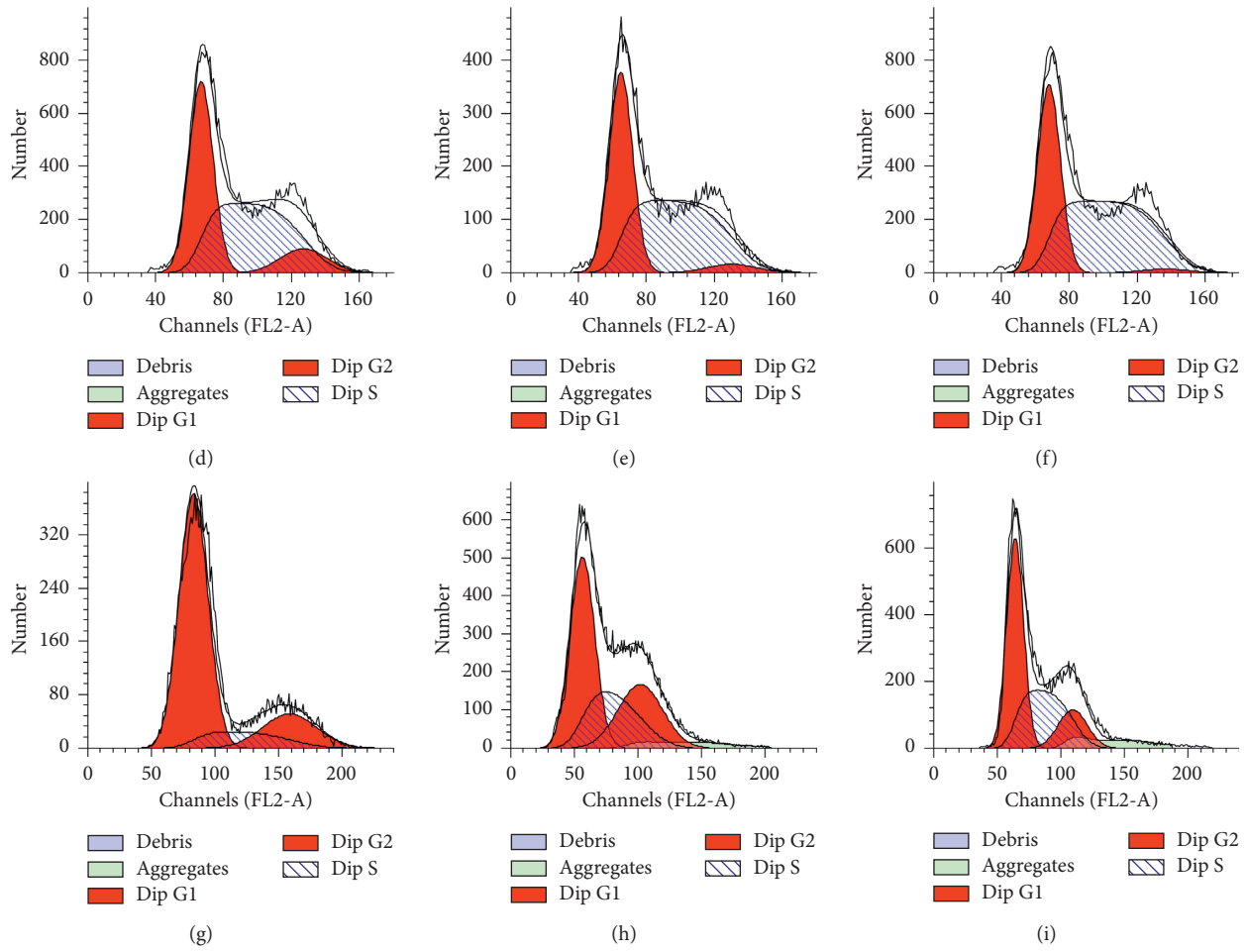


Figure 4: Flow cytometry was performed in A549 ((a) 0 μg/ml; (b) 12.5 μg/ml; (c) 100 μg/ml), H1299 ((d) 0 μg/ml; (e) 12.5 μg/ml; (f) 100 μg/ml), and H2030 ((g) 0 μg/ml; (h) 12.5 μg/ml; (i) 100 μg/ml) cells treated with mangiferin.

Table 1: Effects of different concentrations of mangiferin on apoptosis of lung adenocarcinoma cell lines.

Cell lines	Apoptosis rate (%)		
	0 μmol/L	12.5 μmol/L	100 μmol/L
A549	0.56	8.99	93.13
H1299	0.06	8.97	89.93
H2030	0.14	13.31	53.23

exploration regarding its potent mechanism indicated that mangiferin exerts an apoptosis-inducing and growth-inhibitory effect via the suppression of miR-27a and miR-92.

Which factors are related to mortality for LUAD patients? This is a major question emerging from the rapidly increasing number of lung cancer cases. As an extremely fatal cancer, dysregulation of growth and high metastatic ability limit the probability of curing this disease. Although great achievements have been made within recent decades, the underlying mechanism of LUAD carcinogenesis is still not clear, and patients with LUAD still face a poor prognosis.

Recently, mangiferin has gained much attention for its potential ability against human cancers. Previous studies have shown that mangiferin can induce apoptosis and thus exert an antitumor effect in multiple cancerous cells, including leukemia, nasopharyngeal carcinoma, and HCC cell lines [29, 30]. Specifically, consistent results were also observed in human LUAD A549 cells, in which mangiferin has been reported to inhibit cell proliferation and induce apoptosis by inactivating cdc2-cyclin B and the NF-κB signaling pathway [31]. In addition, treatment with mangiferin strengthens the ability of biological enzymes, such as diphosphate-glucuronosyl transferase, quinone reductase, and glutathione transferase, to break down toxins and lessens genome deterioration in LC-bearing animals [32].

In addition to the latent inhibitory effect of mangiferin on the growth of A549 cells, the discoveries in this paper further suggest that the administration of mangiferin leads to an important inhibition of the proliferation of three subsets of LUAD cells (A549, H1299, and H2030 cells) in a dose- and time-dependent manner. In addition, as detected by Annexin V/PI staining, our results also revealed that mangiferin is able to block the cell cycle and promote cell apoptosis in LUAD cells.

It is universally known that miRNAs exert a crucial influence on the genetic regulation network. MiRNAs have been shown to widely participate in the processes of

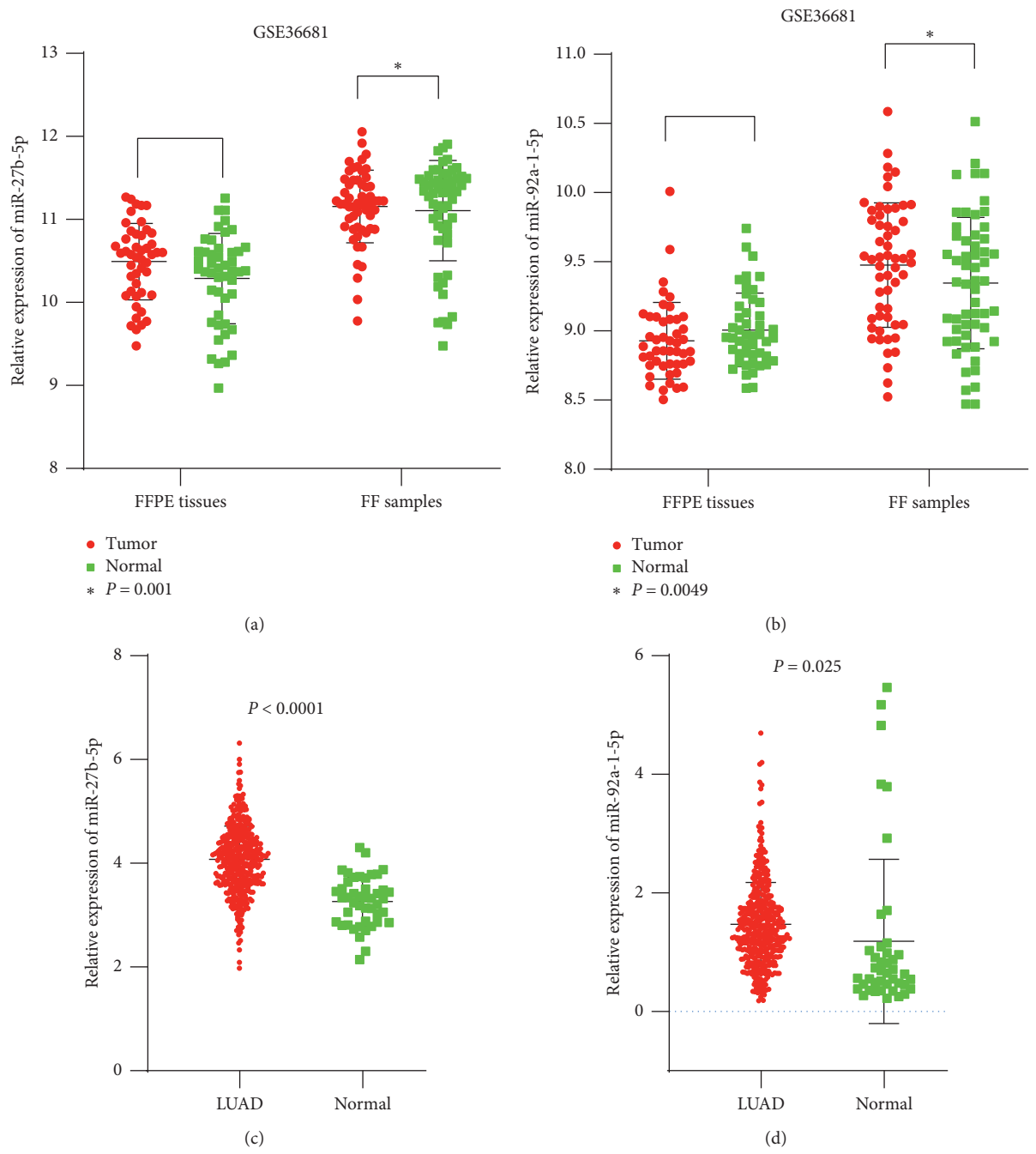


Figure 5: Expression of miR-27b-5p (a, c) and miR-92a-1-5p (b, d) in lung adenocarcinoma tissues from GEO and TCGA database.

tumorigenesis, apoptosis, and differentiation. More than half of human miRNA-related genes in the genome are located in tumor-associated regions or vulnerable sites, and in silico profiling has implied that the level of specific miRNAs might be an indicator to predict tumor differentiation status and stage. It was discovered that mangiferin possesses anticancer effects in glioma by inducing miR-15b expression, and mangiferin has been shown to inhibit proliferation by upregulating miR-182 in prostate cancer cells [10, 33]. Interestingly, the present results indicate that mangiferin treatment specifically represses the expression of miR-92a and miR-27b in LUAD cells.

A study published in 2019 showed that the upregulation of miR-92 clearly reduced the levels of the tumor suppressor gene NF2 in HCT116 and A549 cells and promoted migration, growth, and survival [34]. Additionally, another study examining a total of 2573 patients also suggested that overexpression of miR-92a serves as an unfavorable prognostic indicator in LUAD patients [35]. To our knowledge, miR-27b-5p had been reported as a tumor suppressor in ovarian carcinoma and oral cancer [20, 36], but it is highly expressed in gastric adenocarcinoma and may play a positive role in tumorigenesis and development [37]. However, regrettably, no study has reported the relationship between

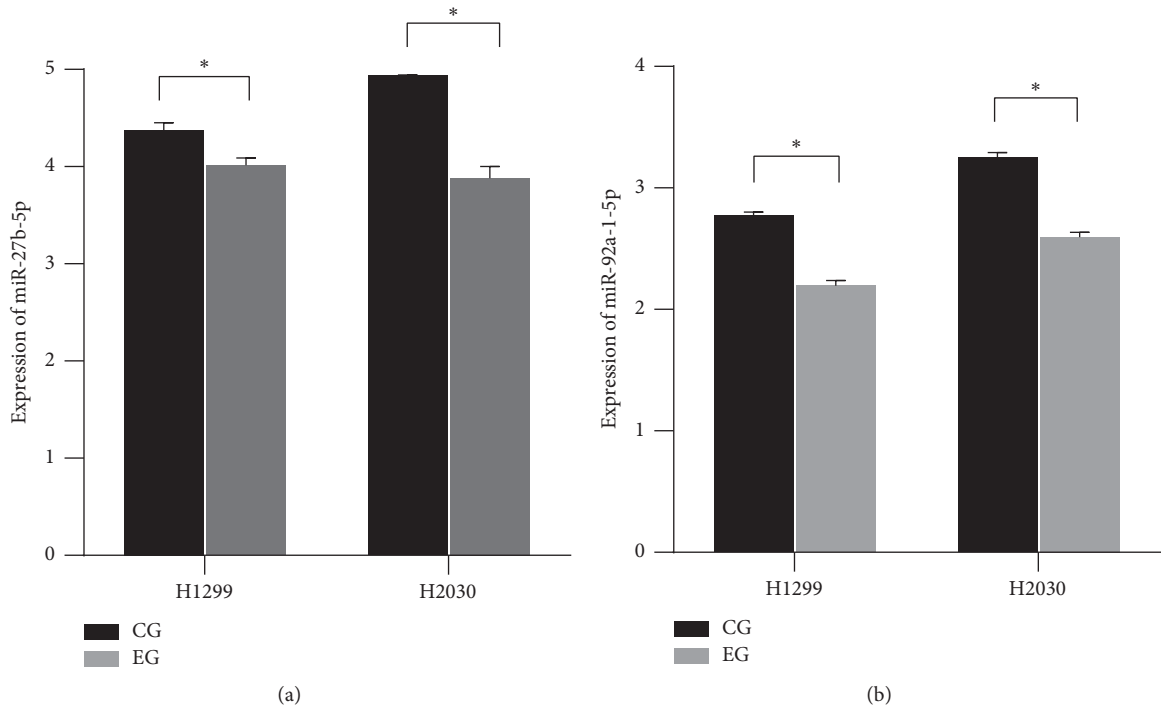
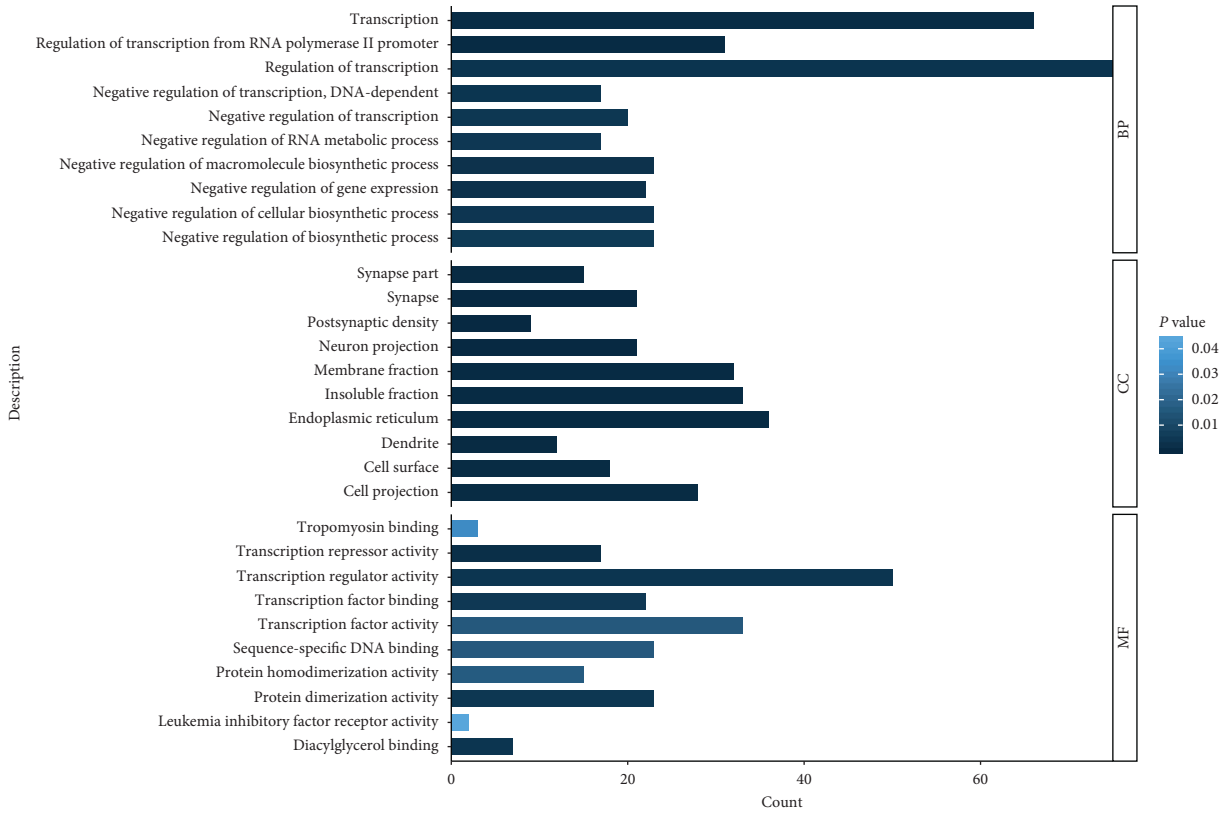
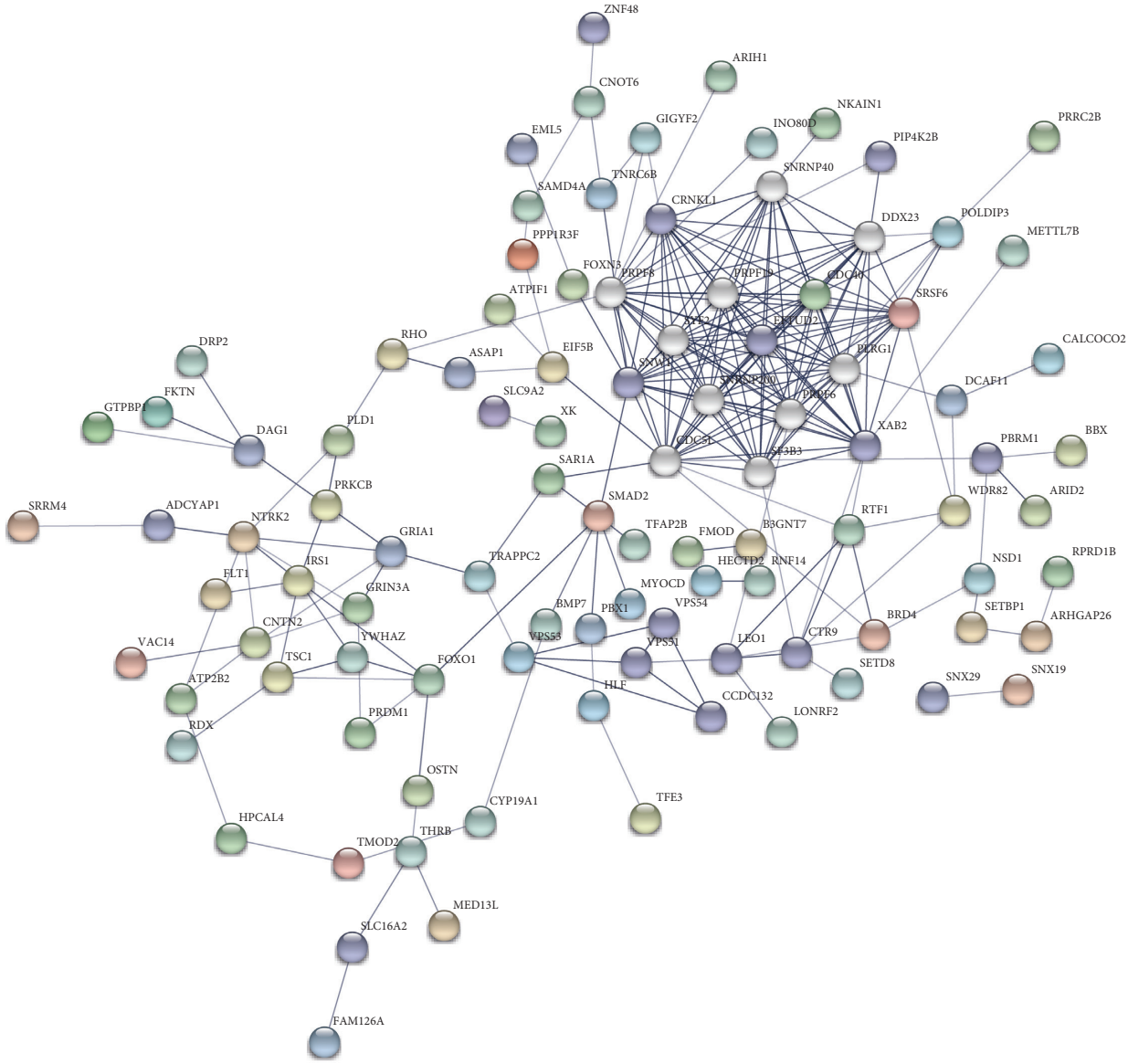


Figure 6: The expression levels of miR-27b-5p and miR-92a-5p in LUAD cells treated by mangiferin (CG: control group; EG: experimental group).



(a)

Figure 7: Continued.



(b)

Figure 7: Integration analysis of potential target genes. (a) Gene Ontology analysis; (b) PPI network of genes (number of nodes: 153, number of edges: 239, PPI enrichment P value: $<1.0e{-}16$).

miR-27b-5p and lung cancer. As determined by data from TCGA and GEO microarrays, both miR-27b and miR-92a are present at a higher level in LUAD tissues than in adjacent tissues. After mangiferin treatment, a marked down-regulation of miR-27b and miR-92a was observed in LUAD cells; thus, we can infer that mangiferin exerts a growth-repressive and apoptosis-inducing effect by directly targeting miR-27b and miR-92a.

However, some shortcomings of this study should be noted. First, the biological function of miR-27b and miR-92a needs to be verified in vitro. In addition, key molecules related to mangiferin-mediated apoptosis or cell cycle arrest were not detected. Moreover, clinical trials should be further performed to confirm whether mangiferin can be used to treat LUAD patients.

In conclusion, we found that mangiferin may negatively regulate the expression of miR-92a and miR-27b to influence not only cancerous growth but also the cell cycle and apoptotic capability of LUAD cells, which indicates that mangiferin is likely to be a hopeful therapeutic agent for LUAD patients. In the future, further experiments are warranted to confirm the current results.

Data Availability

The data used in this study were obtained from open databases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xiao-Jv Chi and Jian-Jun Meng contributed equally to this work.

Acknowledgments

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Research Article

Duchesnea indica Extract Attenuates Coal Fly Ash-Induced Inflammation in Murine Alveolar Macrophages through the NF-Kappa B Pathway

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Duchesnea indica is known as false strawberry, is found in East Asia, and has numerous biological properties. The aim of this study was to investigate the anti-inflammatory effect of *Duchesnea indica* extract (DIE) on coal fly ash- (CFA-) induced inflammation in murine alveolar macrophages (MH-S). Following the induction of inflammation in MH-S cells by CFA, nitric oxide (NO) was measured to evaluate the anti-inflammatory property of DIE. Cell viability and inflammatory gene expression were analyzed using polymerase chain reaction (PCR). The inflammatory pathway in MH-S cells was determined via western blotting and immunofluorescence (IF) analysis. Finally, the major components of the DIE were identified and separated through ultra-performance liquid chromatography (UPLC) and gas chromatography-mass spectrometry (GC-MS) analysis. Our results showed that the DIE dose-dependently inhibited the CFA-induced NO production in MH-S cells. Moreover, the DIE could suppress the CFA-induced proinflammatory mediators, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In addition, the inhibitory effect of the DIE on proinflammatory cytokines, including interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α), was detected with PCR. Moreover, the effect of the DIE on the nuclear factor-κB (NF-κB) pathway in CFA-activated MH-S cells was measured via western blotting. Furthermore, the inhibition of the phosphorylated NF-κB (p-NF-κB) translocation was analyzed using IF assay. The findings of this study indicated that the DIE potentially inhibited the CFA-induced inflammation by blocking the NF-κB inflammatory signaling pathway in MH-S cells and that the DIE might contain favorable anti-inflammatory compounds which may be effective in attenuating lung inflammation.

1. Introduction

Coal fly ash (CFA) is a major environmental factor of air pollution, which is produced regularly due to industrial activities and the use of motor vehicles in the urban areas [1]. CFA consists of liquid and solid particulate matters that vary in size and origin, including coal, combustion particles, and asbestos [1, 2]. Nanoparticles can retain for a long period of time in the atmosphere and easily move in the air. Chronic exposures of CFA can cause accumulation of sediments in the lungs and lead to serious adverse effects, such as inflammation in the alveolar cells [3, 4]. Moreover, nanoparticles are responsible for many respiratory diseases, such as asthma, bronchitis, chronic obstructive pulmonary diseases (COPD), and lung carcinoma [5].

Inflammation is an important biological response to harmful stimuli and the first line of defense in the immune system [6]. Alveolar macrophages (AM) are considered one of the defensive cell populations in the lungs and facilitate regulating lung inflammation and host defense protection against airborne nanoparticles [7]. After CFA invades the airway of the lungs, AM are recruited, resulting in the production of inflammatory mediators and cytokines, which is the initial response of the immune system [8]. CFA-induced excessive proinflammatory cytokines are considered one of the major causes of lung diseases. They can be

regulated by the activation of the transcription factors, such as nuclear factor kappa-B (NF-κB) signaling cascade [9, 10].

Duchesnea indica is a perennial plant that belongs to the Rosaceae family and is widely distributed in Asian countries especially in Bangladesh, China, Japan, and Korea [11]. It has been extensively used as a folk medicine in Asia for its unique biological properties including antioxidative, anti-inflammatory, antimutagenic, and anticancerous properties [12, 13]. Several studies have demonstrated that some phenolic compounds, such as phenolic acid, flavonoids, and ellagic acid, are the main pharmacologically bioactive constituents in *Duchesnea indica* [11]. It has been shown that the *Duchesnea indica* extract (DIE) downregulates cervical cancer through apoptosis and cell cycle arrest [14]. Moreover, previous studies have reported that the DIE protected the skin fibroblast of humans against hydrogen peroxide-induced cytotoxicity and inhibited the growth of human cancer cells in vitro [15]. Thus, traditional medicines have been considered by the patients due to their therapeutic effects and bioactive ingredients [16].

In this study, we aimed to investigate the anti-inflammatory effects of the DIE on CFA-induced inflammation in MH-S cells. Taken together, our results demonstrated that the DIE can prevent the inflammation in CFA-activated MH-S cells, and such activity is mediated by inhibiting the expression of proinflammatory mediators and cytokines.

2. Materials and Methods

2.1. Chemicals and Reagents. The following chemicals and reagents were utilized: Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco's phosphate buffered saline (DPBS) (Welgene, South Korea); CFA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA); Oligo-dT (Bioneer oligo synthesis); COX-2, iNOS, TNF-α, IL-1β, and IL-6 primers (Bioneer, Daejeon, South Korea); dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA); TRIzol reagent (Invitrogen, Carlsbad, CA, USA); Pro-prep (iNtRON biotechnology, South Korea); bovine serum albumin (BSA) (Thermo Fisher Scientific, South Korea); specific antibodies used for western blot, including COX-2, iNOS, p-IKB, p-NF-κB, β-actin, and HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA); secondary antibody used for immunofluorescence (anti-rabbit IgG Fab2, Alexa Fluor 555, Molecular Probes). All other chemicals and reagents were the highest quality available.

2.2. Preparation of the Duchesnea indica Ethanol Extract (DIE). We purchased dried *Duchesnea indica* leaves and stems from the company, ground them into fine coarse powder, and extracted them in 70% ethanol (1-part dry weight of plant to 20-parts of solvent ratio) using a heating mantle (Model: MS-DM604, MTOPS, South Korea) at 80°C for 2 h. Then, the extract was filtered using the Whatman filter paper, followed by extract condensation using the rotary evaporator system (Heating bath-B100 and Rotavapor-R100, BUCHI, Switzerland). Finally, the crude extract was frozen overnight at −70°C and lyophilized using a freeze dryer (Model: FDU-7012, Operon, South Korea) to obtain dry fine powder. The yields were weighed and preserved at −30°C for use in experiments. At the time of the experiment, the powder was dissolved in DMSO with different doses of DIE (12.5, 25, 50, and 100 μg/mL).

2.3. UPLC-QTOF-MS Analysis of DIE. UPLC analysis of the DIE was performed using ACQUITY UPLC^{TM} (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system and coupled to the quadrupole time-of-flight mass spectrometer (Q-TOF Premier^{TM}, Waters Corp., Milford, MA, USA) equipped with an auto-sampler and a UV detector. Briefly, the DIE (2 μL) was injected into the ACQUITY UPLC BEH C18 chromatography column (2.1 × 100 mm × 1.7 μm). The column temperature was fixed at 35°C, and the flow rate was 0.4 mL/min. The chromatographic gradient consisted of mobile phases: (A) water with 1% formic acid and (B) acetonitrile with 1% formic acid. The gradient duration was optimized: 0 min, 10% B; 0–11 min, 10%–90% B; 11–11.5 min, 90%–100% B; 11.5–13.5 min, 100% B; and 13.5–15 min, back to 10% B. The mass spectrometer condition was a negative ion mode with the capillary and cone voltages being 2.3 kV and 50 V, respectively. N_2 was used as a desolvation gas. The source temperature was 100°C, and the desolvation temperature was 350°C. Leucine-enkephalin was used as a reference compound (m/z 554.2615) in the form of a spray.

2.4. GC-MS Analysis of DIE. GC-MS analysis of the DIE was performed using the Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA). The GC-MS device was equipped with a 30 m × 0.25 mm (i.d. DB-5MS) chromatography column and the Agilent 5975C mass selective detector to separate and quantify the compounds of DIE. The extract was injected at 250°C. The temperatures for the transfer line and source were 280°C and 230°C, respectively. The column temperature was set at 70°C as an initial temperature for 1 min and raised to 300°C at a rate of 5°C/min, with duration at a final temperature of 300°C for 30 min. The mass spectrometry was acquired via electron ionization and scan modes. The helium gas was used as a carrier gas with a constant flow rate (1 mL/min).

2.5. Cell Culture. The MH-S cell line, originating from the American Type Culture Collection, was cultured in RPMI supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin sulfate (Welgene, South Korea). The incubation conditions were maintained at 37°C and 5% CO_2 during culture and treatment conditions.

2.6. Nitric Oxide (NO) Assay. NO was measured using the Griess reagent A (0.2% N-ethylenediamine dihydrochloride) and Griess reagent B (2% sulfanilamide in 5% phosphoric acid) reaction methods. Briefly, the MH-S macrophages

were seeded in a 24-well plate and incubated with or without CFA (2.5 μg/mL) in the absence or presence of the DIE (12.5, 25, 50, and 100 μg/mL) at indicated concentrations for 18 h. The Griess reagents (100 μL) were added with cell culture supernatants (100 μL) and incubated for 5 min at normal condition. Then, absorbance was measured in a microplate reader at 540 nm (Versamax, Microplate Reader, Molecular devices, CA, USA).

2.7. Cell Viability Assay. To determine the cytotoxicity of the extract, a cell viability assay was measured as described using 100 μL/well of 3-(4,5-dimethylthiazol-2-yl)-2, and 5-diphenyltetrazolium bromide (MTT) reagent was added to the culture medium [17]. After 2–3 h of incubation at 37°C in 5% CO_2, the supernatants were discarded, and dimethyl sulfoxide (DMSO) (100 μL/well) was added and then incubated at room temperature with shaking for 10 min. Finally, absorbance was analyzed using microplate reader at 560 nm (Versamax, Microplate Reader, Molecular devices, CA, USA).

2.8. RNA Extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and Real-Time qRT-PCR. PCR analysis was conducted in accordance with the previously reported method [18]. The murine macrophages (MH-S) were treated with or without the DIE (12.5, 25, 50, and 100 μg/mL) for 30 min at indicated concentrations, followed by the CFA stimulation (2.5 μg/mL) for 18 h. RNA was collected from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Two micrograms of total RNA was annealed with Oligo-dT (Bioneer, South Korea) for 10 min at 70°C, cooled for 5 min in ice, and then reverse-transcribed using reverse transcriptase premix (Bioneer, South Korea) in 20 μL of reaction mixture for 90 min at 42.5°C on a thermocycler. To inactivate the reverse transcriptase, the reaction was terminated at 95°C for 5 min. RT-PCR was performed using cDNA obtained from RT reaction in a PCR premix (Bioneer, South Korea). Moreover, the PCR products were electrophoresed on agarose gel (1%) stained with ethidium bromide. The band was visualized using ImageQuant LAS 500 (GE Health Care Life Sciences, South Korea). The intensity of the band density was normalized to GAPDH. Real-time PCR was performed using SYBR green. Table 1 shows the RT-PCR and real-time PCR primer sequences.

2.9. Western Blot Analysis. Western blot analysis was conducted in accordance with the previously reported method [10]. MH-S cells were untreated (control group), treated with only CFA (2.5 μg/mL), and treated with the DIE (12.5, 25, 50, and 100 μg/mL) in the presence of CFA (2.5 μg/mL). The proteins were extracted from the cells with the protein extraction solution, Pro-Prep (iNtRON biotechnology, South Korea). Then, the proteins were determined using the protein measurement solution, PRO-MEASURE assay kit (iNtRON biotechnology, South Korea). The cell lysates were then subjected to SDS-PAGE (10%) and transferred onto the PVDF membranes (Millipore, Immobilion-P, Billerica MA, USA). Membranes were blocked for 1 h in 5% (*w*/*v*) skim milk and 0.1% (*v*/*v*) Tween-20 in TBS. Then membranes were incubated with different primary antibodies overnight at 4°C. After washing, 1 h of incubation with horse radish peroxidase- (HRP-) labelled secondary antibody (1 : 3000 dilution, Cell Signaling) at room temperature was performed. Proteins were detected using enhanced chemiluminescence (ECL) solution (Supex, Daegu, Korea). Immunoblots were quantified using the ImageJ software.

2.10. Immunofluorescence Staining. Immunofluorescence (IF) assay was done as described [19]. MH-S cells were washed with DPBS and fixed with 4% paraformaldehyde (PFA) for 10 minutes. Moreover, the cells were permeabilized with 0.2% triton X-100 in TBS (TBST) for 10 minutes and washed with TBST for 5 minutes every three times. Using 2% BSA, the samples were blocked for 1 h, and primary antibody rabbit anti p-NF-κB (Cell Signaling Technology, Danvers, MA, USA) was applied to the cells at 4°C overnight. The cells were washed with TBST for 5 minutes every three times. The samples were incubated with secondary antibody (anti-rabbit IgG Fab2, Alexa Fluor 555, Molecular Probes) for 1 hour in the dark, and after washing with TBST three times, the samples were mounted using ProLong Gold Antifade Reagent with DAPI to visualize the nuclei (Cell Signaling Technology, Danvers, MA, USA) and analyzed via confocal microscopy (ZEISS).

2.11. Statistical Analysis. Data were analyzed by one-way ANOVA or unpaired Student's *t*-test followed by Dunnett's multiple comparison tests using the GraphPad Prism software. Data are presented as mean ± SEM. The statistical significance is denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ or ns = not significant.

3. Results

3.1. Active Compositions of the DIE Based on the UPLC-QTOF-MS and GC-MS Analyses. Active compounds in the DIE were determined via ultra-performance liquid chromatography (UPLC) analysis. Based on the results of the UPLC-QTOF-MS analysis, the main active compound of the DIE was ellagic acid (Figure 1). A previous study reported that phenolic compounds, such as ellagic acid, phenolic acid, and flavonoids, are the major active compounds in *Duchesnea indica* [20–22]. Moreover, based on the results of the gas chromatography-mass spectrometry (GC-MS) analysis, the major components were as follows: gamma-sitosterol; hexadecanoic acid; linoelaidic check spacing acid; octadecanoic acid; 9, 12, 15-octadecatrienoic acid; and 2, 3-dihydro-3, 5-dihydroxy-6-methyl-(4H)-pyran-4-one (Table 2).

3.2. DIE Protects CFA-Induced Nitric Oxide Production and Cell Death in MH-S Macrophages. Nitric oxide (NO) is an important mediator in the inflammatory condition, and excessive production of NO contributes to inflammatory

Table 1: Primer used for RT-PCR and real-time PCR analysis.

RT-PCR	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
COX-2	CACTACATCCTGACCCACTT	ATGCTCCTGCTTGAGTATGT
iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGCCTCGTGGCTTTGG
IL-6	GTACTCCAGAAGACCAGAGG	TGCTGGTGACAACCACGGCC
TNF-α	TTGACCTCAGCGCTGAGTTG	CCTGTAGCCCACGTCGTAGC
IL-1β	CTGTGGAGAAGCTGTGGCAG	GGGATCCACACTCTCCAGCT
GAPDH	CACTCACGGCAAATTCAACGGCAC	GACTCCACGACATACTCAGCAC
Real-time PCR		
COX-2	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
iNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
IL-6	TCCAGTTGCCTTCTTGGGAC	GTGTAATTAAGCCTCCGACTTG
TNF-α	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
GAPDH	CACTCACGGCAAATTCAACGGCAC	GACTCCACGACATACTCAGCAC

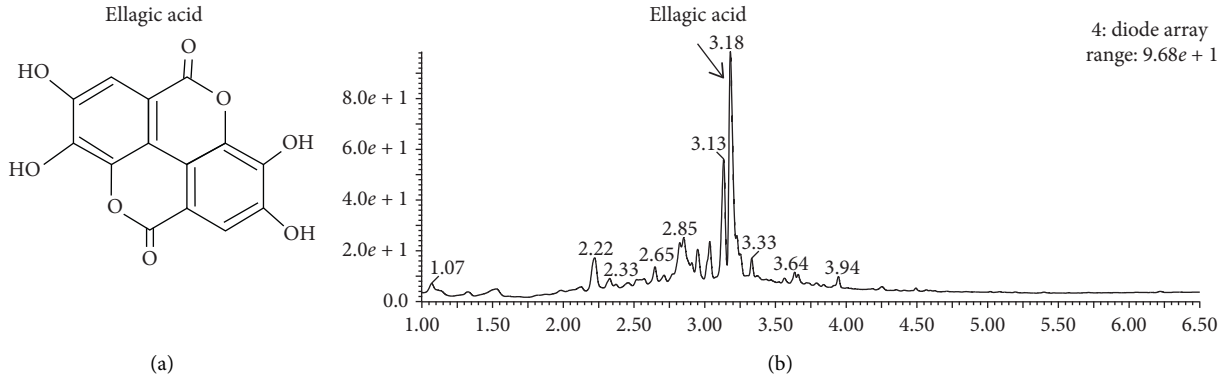

Figure 1: Chemical compositions of the *Duchesnea indica* extract (DIE). (a) Chemical structure of ellagic acid. (b) Ellagic acid was identified via UPLC-QTOF-MS analysis as a major compound, and the retention time was 3.18 min.

Table 2: Major compounds in the *Duchesnea indica* extract identified via GC-MS analysis.

Compounds	Area (%)
2,3-Dihydro-3,5-dihydroxy-6-methyl-(4H)-pyran-4-one	20.37
Hexadecanoic acid	18.04
9,12,15-Octadecatrienoic acid	11.1
Gamma-sitosterol	7.25
Octadecanoic acid	5.52
Linoelaidic acid	4.54

diseases [23, 24]. In this study, the levels of NO in response to CFA in MH-S murine AM were measured using the Griess reaction method. The DIE potently inhibited the NO induction in a dose-dependent manner (Figure 2(a)). MTT assay was performed to determine the cell viability assay, and the results showed that the DIE did not affect the cell viability at the used concentration (Figure 2(b)). These results demonstrated that the DIE inhibited the NO production dose-dependently, and the used concentrations did not show cytotoxicity.

3.3. Preventive Effect of the DIE on CFA-Induced Proinflammatory Cytokines in MH-S Macrophages. Pretreatment of MH-S with the DIE for 30 min decreased the levels of CFA-induced proinflammatory mediators and cytokines. The mRNA levels of proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and proinflammatory cytokines including interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) were analyzed using RT-PCR to investigate the anti-inflammatory properties of the DIE. The results showed that proinflammatory mediators and cytokines were dose-dependently reduced at the mRNA level (Figures 3(a)–3(f)). This finding suggested that the DIE downregulated the CFA-induced inflammatory cytokines and significantly reduced the mRNA levels mainly at concentrations of 50 and 100 μg/ml.

3.4. DIE Ameliorates the CFA-Induced mRNA Expression of Proinflammatory Cytokines in MH-S Macrophages. To confirm the results of the RT-PCR on the proinflammatory mediators and cytokines in MH-S cells, the mRNA expressions of the proinflammatory mediators were investigated and the proinflammatory cytokines were analyzed with real-time PCR. The mRNA expressions of the proinflammatory mediators and cytokines were noticeably dose-dependently downregulated with the DIE treatment (Figures 4(a)–4(e)). Taken together, the real-time PCR results suggested that the DIE reduced the inflammation in a concentration-dependent manner.

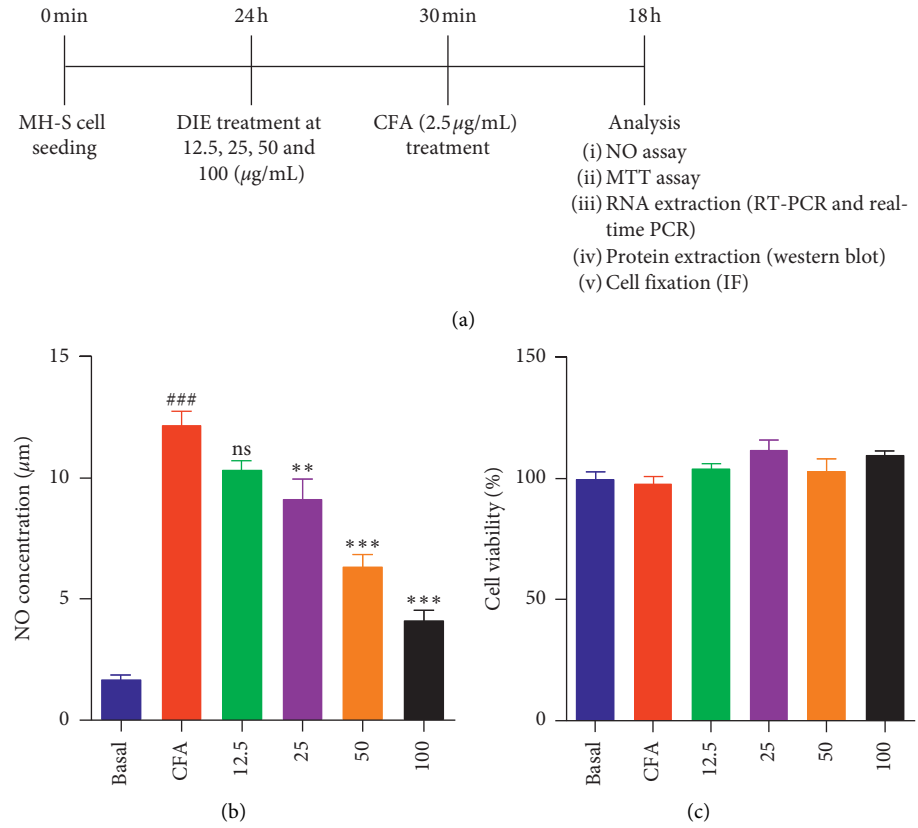

Figure 2: Effect of the *Duchesnea indica* extract (DIE) on coal fly ash- (CFA-) induced NO production and cell viability in murine alveolar macrophages (MH-S). (a) Schematic diagram of this study. The cells were in six groups including the basal (control) group, only CFA (2.5 μg/mL) group, and CFA with the DIE (12.5, 25, 50, and 100 μg/mL) group. Cells were treated with the above concentrations of the DIE for 30 min prior to the CFA treatment (2.5 μg/mL) and incubated for 18 h. (b) NO level was determined using the Griess reagent method. (c) Cell viability assay was performed using the MTT reagent method. The cells were seeded in a 24-well plate. All values were expressed as standard error of mean ± (SEM) from three independent experiments. $^{###}p < 0.001$ when compared with basal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the only CFA-treated group.

3.5. DIE Inhibits the Activation of NF-κB Signaling in CFA-Treated MH-S Macrophages. The effects of the DIE on nuclear factor-κB (NF-κB) signaling were measured as a transcription factor has an important role in inflammation. The activation with CFA leads to the inflammatory pathway, wherein NF-κB signaling is a key axis in the inflammatory mechanism [1, 9]. Treatment with CFA significantly induced the phosphorylation of NF-κB and inhibitor of kappa-B (IκB), whereas the pretreatment of the DIE significantly inhibited the phosphorylation of NF-κB and IκB in MH-S cells (Figures 5(d) and 5(e)). This result suggested that the pretreatment with the DIE markedly downregulated the phosphorylation of the CFA-induced NF-κB and IκB activation in MH-S cells.

3.6. DIE Inhibits the Translocation of NF-κB in CFA-Treated MH-S Macrophages. To find out whether the anti-inflammatory activities of the DIE are mediated by the NF-κB signal transduction pathway in CFA-activated MH-S cells, the translocation of the activated p-NF-κB from the cytoplasm to the nucleus was determined via immunofluorescence (IF) assay. The treatment with CFA increased the translocation of NF-κB from the cytoplasm to the nucleus, whereas the treatment with highest dose of DIE (100 μg/mL) significantly suppressed the nuclear translocation of p-NF-κB in activated MH-S cells (Figure 6). Bay-11 was used as an NF-κB inhibitor. The results of the IF assay indicated that the anti-inflammatory properties of the DIE were associated with its inhibitory effects on the phosphorylation of the NF-κB signal pathway (Figure 7).

4. Discussion

Duchesnea indica has been widely used as a natural medicine in Asia especially in Bangladesh, China, Korea, and Japan for the prevention and treatment of numerous diseases, such as tissue inflammation, leprosy, congenital fever, and mainly cancer [12, 14]. Earlier studies have shown that its anti-inflammatory agents can suppress transcription factors, such as nuclear factor-κB (NF-κB) and function as a key regulator in inflammatory cascade [2, 7, 9]. In the present study, we examined the effects of *Duchesnea indica* on CFA-induced inflammation in the MH-S cell line.

Pharmacological studies have demonstrated that phenolic ingredients are the main bioactive compounds in the

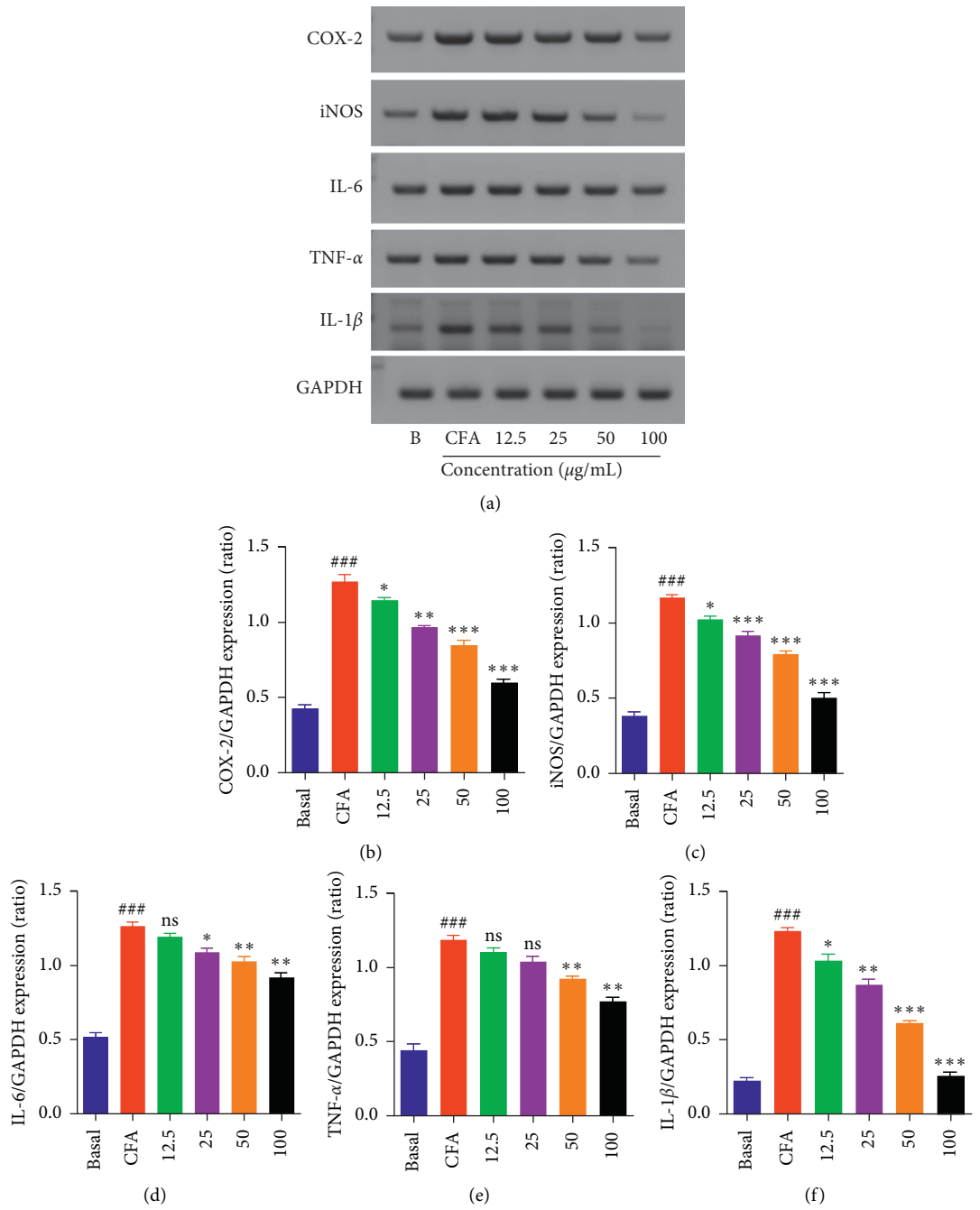

Figure 3: Effect of the DIE on CFA-induced proinflammatory mediators and cytokines in MH-S cells analyzed via RT-PCR. (a) After 18 h of incubation with CFA (2.5 μg/mL), the mRNA levels of the proinflammatory mediators (COX-2 and iNOS) and proinflammatory cytokines (IL-6, TNF-α, and IL-1β) and GAPDH (housekeeping gene) were determined via reverse transcription-polymerase chain reaction (RT-PCR). (b–f) Densitometric analysis of relative mRNA expression levels which were quantified using the ImageJ program. The cells were seeded in a 6-well plate, and the doses of DIE were used as 12.5, 25, 50, and 100 μg/mL. All values were expressed as standard error of mean ± (SEM) from three independent experiments. $^{###}p < 0.001$ when compared with the basal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the only CFA-treated group.

DIE [11]. Ellagic acid was indicated as the main compound of the DIE via the UPLC-QTOF-MS analysis. It is a phenolic compound and has been reported to exert a variety of biological properties, such as antioxidant, anti-inflammatory, and anticoagulant effects [25, 26]. Moreover, ellagic acid has also been shown to reverse hepatic damage by inhibiting the NF-κB signaling pathway [27]. Previous findings are consistent with our study and suggest that ellagic acid could be one of the major active components responsible for any anti-inflammatory activity. The major compounds in the DIE were determined via GC-MS analysis: 2, 3-dihydro-3, 5-dihydroxy-6-methyl-(4H)-pyran-4-one; hexadecanoic acid;

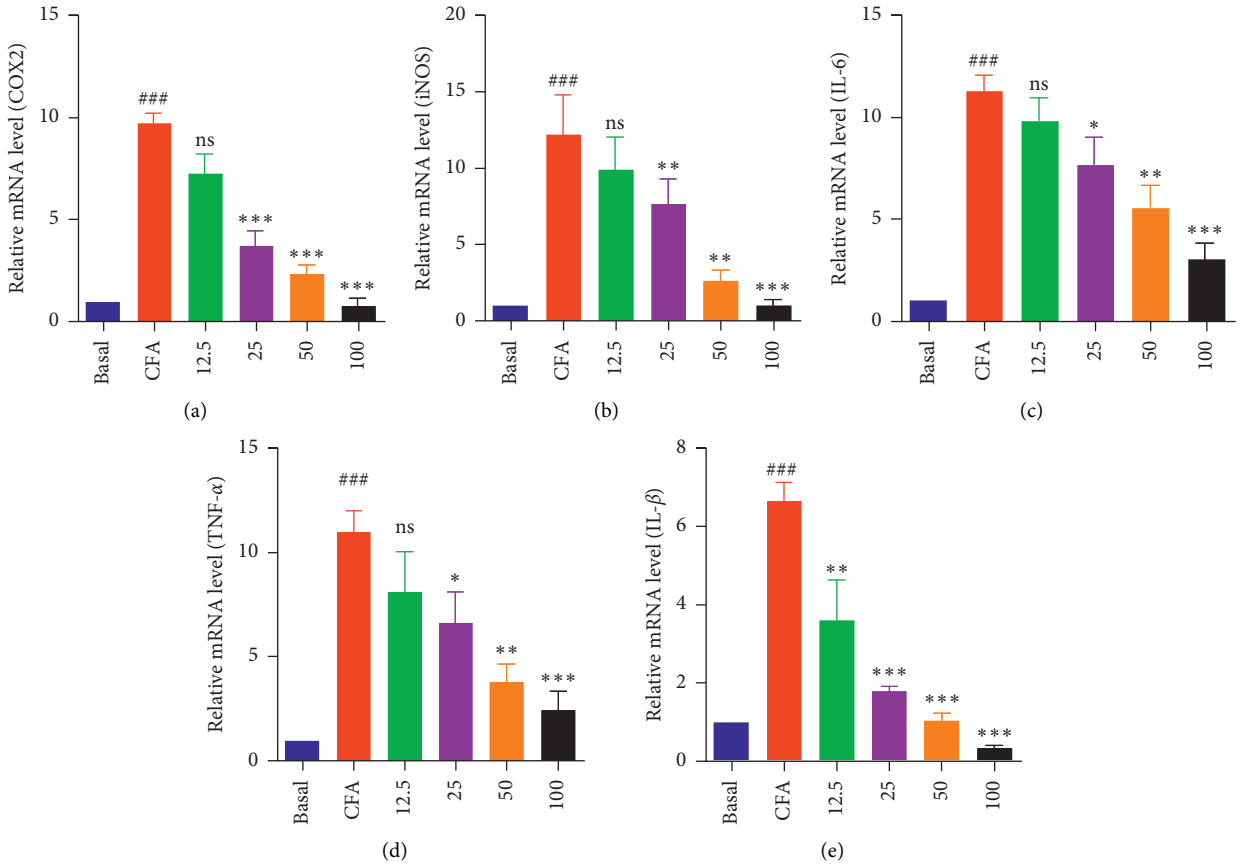

Figure 4: Effect of the DIE on CFA-induced mRNA expression in MH-S cells measured by real-time PCR. (a–e) After 18 h of incubation with CFA (2.5 μg/mL), the mRNA levels of COX-2, iNOS, IL-6, TNF-α, and IL-1β were analyzed via quantitative real-time PCR. GAPDH was used as a control gene. The cells were seeded in a six-well plate, and the doses of DIE were used as 12.5, 25, 50, and 100 μg/mL. All values were expressed as standard error of mean ± (SEM) from three independent experiments. $^{###}p < 0.001$ when compared with basal group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the only CFA-treated group.

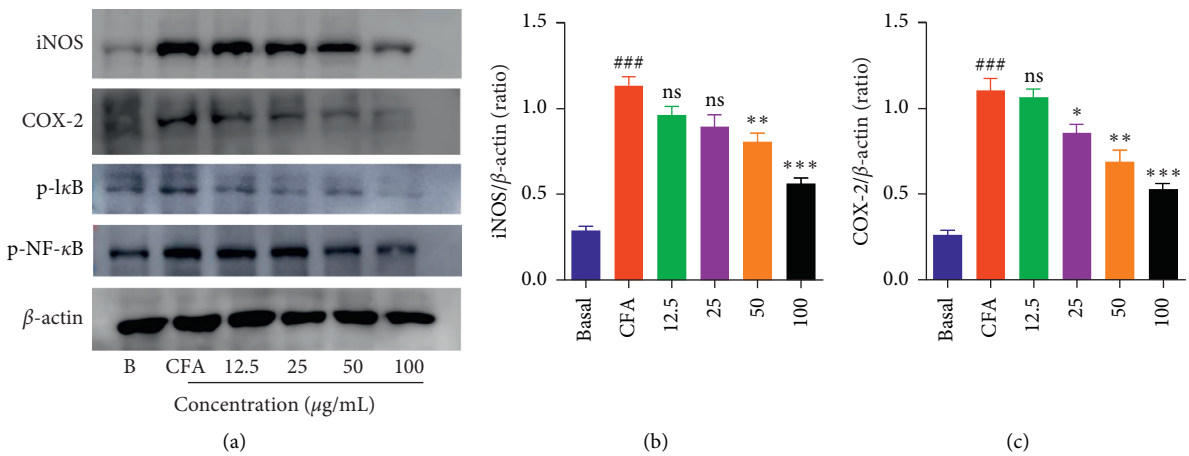

Figure 5: Continued.

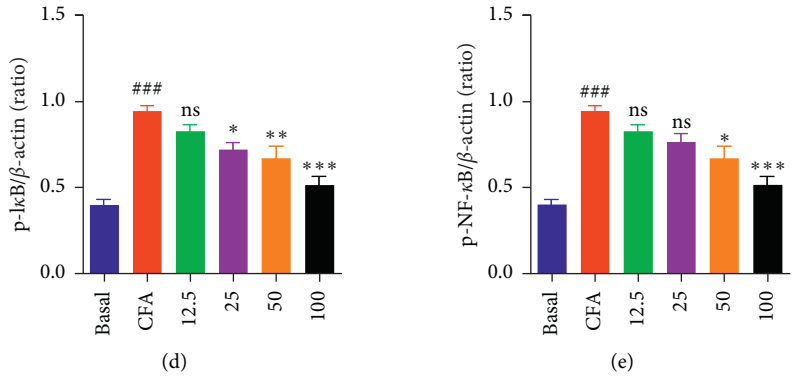

Figure 5: Effect of the DIE on CFA-induced NF-κB phosphorylation in MH-S cells. (a) After 18 h of incubation with CFA (2.5 μg/mL), the protein levels of iNOS, COX-2, p-IκB, and p-NFκB were determined using western blot. β-Actin was used as a loading control. (b–e) Densitometric analysis of protein expression levels which were measured using the ImageJ software. The cells were seeded in a six-well plate, and the doses of DIE were used as 12.5, 25, 50, and 100 μg/mL. All values were expressed as standard error of mean ± (SEM) from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with the only CFA-treated group.

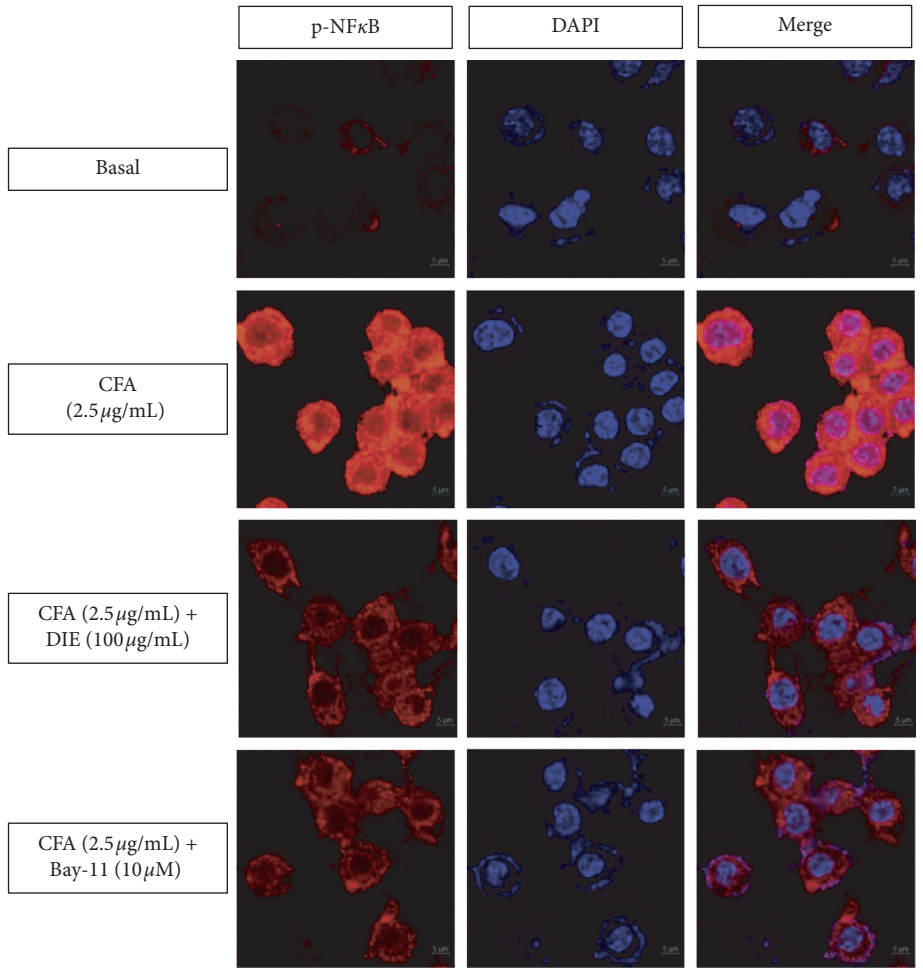

Figure 6: Effect of the DIE on CFA-induced NF-κB translocation in MH-S cells. The cells were seeded on coated cover slip in a six-well plate, and the groups were as follows: basal group, only CFA (2.5 μg/mL)-induced group, CFA with the DIE (100 μg/ml) group, and CFA with Bay-11 (inhibitor of p-NF-κB) group. The cells were treated with the DIE and Bay-11 (10 μM) for 30 min prior to the CFA treatment (2.5 μg/mL) and were incubated for 18 h. The nuclear translocation of p-NF-κB was analyzed via immunofluorescence staining. The samples were mounted using ProLong™ Gold Antifade Reagent with DAPI to visualize the nuclei (blue). Stained cells were analyzed via confocal microscopy (ZEISS) at 1000x magnification.

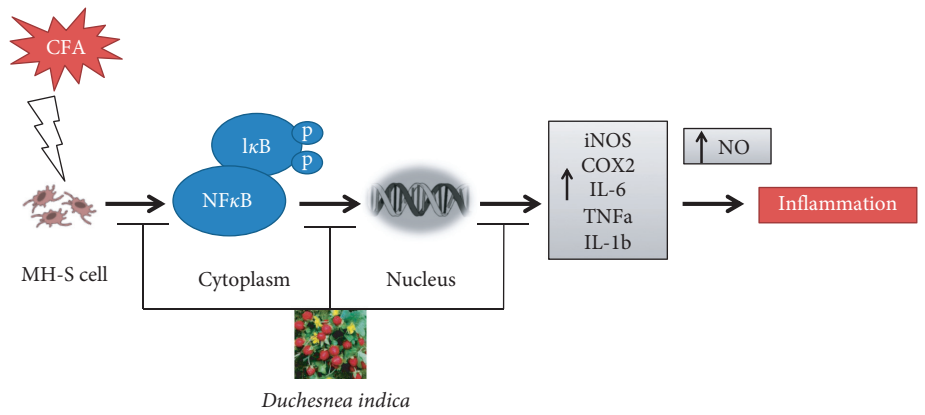

Figure 7: The mechanism of the DIE in CFA-induced inflammation in MH-S cells.

9,12,15-octadecatrienoic acid; gamma-sitosterol; octadecanoic acid; and linoelaidic acid. Although the DIE has anti-inflammatory effects on CFA-induced inflammation in MH-S cells, further research is required to investigate the immune modulatory properties of the DIE in an animal model.

Particulate matter (PM) found in polluted air is becoming a major cause for health problems [28]. It has been reported that chronic exposure of PM is related to chronic inflammatory diseases especially severe lung diseases including chronic respiratory diseases, COPD, asthma, and mainly lung cancer [1, 29]. Inflammatory disorders cause excessive production of proinflammatory mediators and cytokines, which are the critical factors of many pathological and clinical manifestations [30]. Macrophages are the immune cells found in the immune regulatory system and responsible for upregulating the generation of inflammatory mediators.

Nitric oxide (NO) is generated by the iNOS in activated macrophages. NO is a small signaling molecule with essential roles in numerous body functions, but unregulated NO production can cause pathophysiological activities in the airway system [31, 32]. Such disturbances can lead to airway narrowing and lung hypersensitivity. The results showed that the DIE reduced NO production in CFA-induced MH-S cells, whereas NO production was markedly elevated in the only CFA-treated group. COX-2, which is another important proinflammatory mediator, plays a critical role in diverse inflammatory diseases [33]. In this study, it was found that the DIE suppressed the mRNA and protein expression levels of inflammatory mediators COX-2 and iNOS in CFA-activated MH-S cells.

Activated macrophages also generated other important proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, which are responsible for chronic inflammatory diseases and especially respiratory pathology [34]. IL-6 and TNF-α are the critical mediators of sepsis, and their uncontrolled regulation can cause cell death and DNA damage depending on the severity of cytokine production [9]. IL-1β increased the infiltration of inflammatory cells and mediated the activation of inflammatory cascade [35]. In the present study, the DIE inhibited the production of the IL-6, TNF-α, and IL-1β mRNA expressions in CFA-activated MH-S cells. In this study, the DIE also suppressed the protein expression of iNOS and COX-2, suggesting that the DIE has anti-inflammatory properties.

It is well established that the transcription factor, NF-κB, is a key regulator for inflammation in stimulated macrophages. In normal conditions, NF-κB is stable in the cytoplasm; however, upon CFA stimulation, activated cells result in the phosphorylation of IκB. Hence, the NF-κB is disrupted to induce the phosphorylation, and activated NF-κB translocates into the nucleus from the cytoplasm [36, 37]. Thus, the p-NF-κB then promotes the expression of proinflammatory mediators and cytokines. It has been previously shown that phosphorylated NF-κB increases the transcription of inflammatory genes [38]. The western blot results demonstrated that the DIE dose-dependently reduced the CFA-stimulated IκB and NF-κB phosphorylation in the cytoplasm. Moreover, the results of the immunofluorescence staining indicated that CFA increased the p-NF-κB translocation in the nucleus, where the DIE (100 μg/mL) and Bay-11 (10 μM) markedly inhibited the p-NF-κB translocation from the cytoplasm to the nucleus. Based on our study, we speculated that the DIE might inhibit the phosphorylation of IκB and NF-κB resulting in the suppressive activity of the proinflammatory cytokines. In our study, only in vitro experiment was carried out, but in vivo excrement should be done to determine the exact mechanism of action of DIE as an anti-inflammatory agent.

5. Conclusion

In summary, the current study demonstrated the anti-inflammatory properties of the DIE in the CFA-induced MH-S cell line. The obtained data provide important information about CFA-activated inflammatory responses and suggest that the DIE could inhibit the expression of proinflammatory mediators and cytokines in MH-S cells. These findings demonstrate that the DIE could be used as a promising bioactive functional food for immune modulation, especially in the treatment of lung inflammatory diseases.

Data Availability

All data generated during this study are included within this manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Relaxant Effect of *Urginea maritima* on Tracheal Smooth Muscle Mediated by the Effect on Beta-2 Adrenergic, Muscarinic Receptors and Calcium and Potassium Channels

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Urginea maritima (*U. maritima*) showed anti-inflammatory, antioxidant, antibacterial, diuretic, vasodilatation, and wound-healing effects on fungal infections, cardiac disorders, digestive disorders, rheumatoid disease, and respiratory disorders such as bronchitis, bronchial nosocomial infections, and severe cough. To examine the bronchodilatory effect of *U. maritima*, the relaxant effect of its extract on rat tracheal smooth muscle (TSM) and its possible mechanism was examined in this study. Male Wistar rats' TSM were divided into eight groups ($n = 8$ in each group). Four of these groups were TSM tissues, contracted with KCl (60 mM) incubated with atropine, glibenclamide, and indomethacin and nonincubated TSM, while the other four groups were TSM tissues contracted with methacholine (10 μM) for 5 min, incubated with propranolol, chlorpheniramine, and diltiazem and nonincubated TSM. Cumulative concentrations of *U. maritima* extract (12.5, 25, 50, 100, 20, and 400 μg/ml) were then added to organ bath every 5 min. Theophylline (0.2, 0.4, 0.6, and 0.8 mM) as positive control and saline (1 ml) as negative control were also examined in nonincubated tissues. A concentration-dependent relaxant effect of *U. maritima* on non-incubated TSM contracted with KCl (60 mM) or methacholine (10 μM) ($p < 0.01$ and $p < 0.001$) was observed. The relaxant effects of *U. maritima* extract in the incubated tissues with glibenclamide, propranolol, diltiazem, atropine, and chlorpheniramine were significantly lower than those in the nonincubated tissues ($p < 0.05$ to $p < 0.001$). EC_{50} values of *U. maritima* extract in the incubated TSM with glibenclamide, propranolol, diltiazem, and atropine were significantly higher than those in the nonincubated tissues ($p < 0.05$ for diltiazem-incubated tissues and $p < 0.001$ for other cases). *U. maritima* extract displayed considerable relaxant effect on TSM comparable to the effect of theophylline. Beta-2 adrenoceptor stimulation and muscarinic receptor inhibition as well as potassium opening and calcium channels blocking effects are the possible mechanisms for the relaxant effects of the plant.

1. Introduction

One of the most important chronic inflammatory diseases in the world is asthma with considerable morbidity. Asthma is characterized by pathological changes in the lung, like increased mucosa secretion, airway hyperresponsiveness, infiltration of inflammatory cells, and smooth muscle hyperplasia [1]. Over the past 30 years, there has been an increase in the number of patients with asthma, and 250,000 people die from this disease each year. The treatment of this disease is very costly and the direct and indirect costs of the asthma are globally on the rise [2]. The precise mechanism of asthma pathophysiology and the role of biochemical intermediates involved in asthma are not yet known, but,

based on available information, leukotrienes, prostaglandins, histamine, nitric oxide, and type II immune response cytokines are among the most important mechanisms involved in the pathophysiology of asthma. These mechanisms cause airway inflammation as well as bronchospasm in asthmatic patients [3]. The adrenoceptor and cholinergic pathways affected the airway smooth muscle tone. Cholinergic control is performed by the vagal reflex and through stimulatory receptors located below the mucous membranes of the large respiratory tract and upper respiratory tract. The stimulation of these receptors by inhaled stimulants or inflammation causes obstruction of large airways. Androgen intermediates like histamine and prostaglandins may cause bronchial smooth muscle contraction directly or reflexively through stimulation of receptors [4].

The tendency to use treatments with minimizing side effects such as the use of medicinal herbs which may cause a decrease in drug resistance has increased in recent years [5]. *Urginea maritime* (*U. maritima*) is a flowering plant of the Asparagaceae family and the Scilloideae subfamily, known as squill, sea squill, and onion, growing in the Mediterranean, North Africa, and India [6, 7]. *U. maritima* contains a large number of glycosides, type bufadienolide, where scillaren A is an important component of all glycosides. *U. maritima* also contains an aclicon called scillaridin A and small amounts of other cardiac glycosides, such as glucoscillaren A, proscillaridin A [8], other flavonoids, fatty acids, anthocyanins, and related carbohydrates [9, 10]. *U. maritima* plant has been widely used in cardiac disorders and fungal infections and as a diuretic agent, and the fresh *U. maritima* extract is more active than the dried fruits [11]. Constituents of methanolic extract of *U. maritima* exhibited antioxidant and antibacterial activity [12]. A previous document exhibited biological activity of *Urginea* species [13]. The plant also displayed antibacterial activity, inhibition of the growth of an ascites tumor [14], and cardiotonic and diuretic effects [15]. The potent digitalis-like cardiac effect of the plant was described for centuries [16], and it showed heart stimulatory and diuretic effects [17]. Fresh bulbs of this species are used to accelerate wound-healing, as well as in digestive disorders and rheumatoid disease [18]. The effect of this plant on the treatment of cancer was also shown [19]. It showed cytotoxicity against human breast carcinoma cells (MCF-7) in vitro [20]. *U. maritima* extract also caused peripheral vasodilatation in anesthetized rabbits [21] and it showed similar effect to that of digitalis on the heart. *U. maritima* is traditionally used to treat bronchitis, bronchial nosocomial infections, severe cough, and edema [22, 23].

Therefore, to examine the bronchodilatory effect of *U. maritima*, this study sought to investigate the relaxant effect mechanisms of action of its extract on tracheal smooth muscle (TSM) in Wistar rats.

2. Materials and Methods

2.1. Preparation of the Extract. *U. maritima* was purchased from a market in Mashhad, Iran, in October 2018 and identified by Dr. Rakhshandeh, Pharmacological Research Center of Medicinal Plants and Department of Pharmacology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. *U. maritima* extract was prepared by peeling, weighed (50 g), and soaked in 70% ethanol (ethanol 96°, Taghtir Khorasan Co., Iran) at 40°C for 72 hours while shaking constantly. The extract was dried by rotary evaporator at 50°C to obtain a yield of 12% and the required concentrations were prepared.

2.2. Animals and Experimental Groups. Sixty-four male Wistar rats (weight, 200–250) were kept in a standard condition, 22 ± 2°C temperature, 12 h light/dark cycles, and free access to standard diet and tap water in the Animal House, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. The study was approved by the Ethics Committee of Mashhad University of Medical Sciences (#961800). All experiments on animals were done according to National Laws regarding the use and care of laboratory animals. Animals were divided into eight groups ($n = 8$ in each group) as shown in Table 1.

2.3. Tissue Preparation. The rats were sacrificed after anesthetizing by 1.6 g/kg intraperitoneal (i.p.) administration of urethane and their chests opened. Tracheal rings of rats containing three cartilages were prepared from the middle section of trachea as previously described [24] and mounted in a 10 ml organ bath containing Krebs-Henseleit solution supplied with 95% O_2 and 5% CO_2 and tissue responses were measured using an isometric transducer (MLT0202, AD Instruments, Australia) connected to a power lab system (Power Lab 8/30, ML870, AD Instruments, Australia) exactly as previously described [24–26].

2.4. Examination of Smooth Muscle Relaxant Effect of Plant Extract. TSM was contracted by KCl (60 mM) (Merck Chemical Ltd., Germany) or methacholine (10 μM) (Sigma Chemical Ltd., UK) [26]. It was well established that KCl contracts tracheal smooth muscles by depolarizing the smooth muscle cells and methacholine contracts them by muscarinic receptor stimulation [24, 26]. After 5 minutes, cumulative concentrations of extract of *U. maritima* (12.5, 25, 50, 100, 20, and 400 μg/ml) [27] and theophylline (0.2, 0.4, 0.6, and 0.8 mM) as positive control or saline (1 ml) as negative control were added to organ bath every 5 minutes [25, 26].

The reduction of contraction produced contractile agents (KCl or methacholine) due to the fact that each concentration of *U. maritima* extract and theophylline in proportion to maximum contractile response was calculated and considered as percent relaxation response [24]. The relaxation concentration response curves were prepared, and the extract concentration causing 50% of maximum relaxation effect (EC_{50}) was measured from concentration response curve as previously defined [24–26, 28–31]. In incubated tissues with rightward shift in the concentration-response curve of the relaxant effect of the plant, the concentration ratio minus one (CR-1) was

Table 1: Experiment groups. Trachea of incubated groups subjected to different channel blocker or antagonists in organ bath first followed by contraction of TSM by KCl or methacholine after 10 min.

Contractile agent	Condition	Incubating agent	Mechanisms	n
KCl (60 mM)	Nonincubated tissues	—	—	$n = 8$
	Incubated tissues	Atropine (1 μM)	Muscarinic receptor inhibition	$n = 8$
		Indomethacin (1 μM)	Cyclooxygenase inhibition	$n = 8$
		Chlorphenamine (1 μM)	Histamine (H1) receptor inhibition	$n = 8$
Methacholine (10 μM)	Nonincubated tissues	—	—	$n = 8$
	Incubated tissues	Diltiazem (5 μM)	Calcium channel blocking	$n = 8$
		Glibenclamide (1 μM)	Potassium channel opening	$n = 8$
		Propranolol (1 μM)	B_2-adrenoceptor stimulation	$n = 8$

also estimated as (EC_{50} in the incubated tissues/EC_{50} in nonincubated tissues)-1.

In two groups, the relaxant effect of *U. maritima* extract was examined on KCl- or methacholine-contracted on nonincubated TSM to evaluate the possible relaxant effect of the plant and its possible effect on potassium channels and muscarinic receptors, respectively. In addition, in six groups, the relaxant effects of the extract on incubated TSM tissues with atropine, glibenclamide, and indomethacin, contracted by KCl, as well as incubated TSM with propranolol, chlorpheniramine, and diltiazem, contracted by methacholine, were examined to evaluate different possible mechanisms responsible for the relaxant effect of *U. maritima* extract as described in Table 1. The duration of the examination of the relaxant effect of the extract including mounting the tracheal ring in the organ bath, tissue equilibration, TSM contraction, and evaluation of the relaxant effect in each experiment was about 100 minutes.

2.5. Statistical Analysis. Statistical comparisons were performed using InStat software. The data was presented as mean ± standard error of the mean (SEM). Comparisons were performed using ANOVA followed by Tukey's multiple comparisons test and $p < 0.05$ was considered as a significant criterion.

3. Results

3.1. The Relaxant Effect of U. maritima Extract on TSM Contraction Induced by Methacholine in Nonincubated and Incubated Tissues. Concentration-dependent and significant relaxant effects of the extract of *U. maritima* and theophylline were seen on TSM contracted by methacholine ($p < 0.05$ for the second extract concentration and $p < 0.001$ for all theophylline and higher extract concentrations).

The relaxant effects of two higher concentrations of *U. maritima* extract (200 and 400 μg/ml) were significantly less than the relaxant effects of the two higher concentrations of theophylline ($p < 0.05$ for both cases) (Figure 1(a)).

Different concentrations of *U. maritima* extract showed significant relaxant effects on TSM in incubated tissue with glibenclamide ($p < 0.001$ for 5 last concentrations). However, in incubated tissues with glibenclamide, the relaxant effects of 100 and 200 μg/ml concentrations of the extract were significantly lower than those in the nonincubated TSM ($p < 0.001$ and 0.05 for 100 and 200 μg/ml concentrations, respectively) (Figure 1(b)). EC_{50} values of the *U. maritima* extract for its relaxant effect in incubated TSM with glibenclamide were significantly higher than those in nonincubated tissues (Figure 2(a)).

A rightward shift in the concentration-response relaxation curve of the *U. maritima* extract was observed; in glibenclamide-incubated TSM compared to nonincubated tissues, a maximum response was achieved. The (CR-1) value of the extract in incubated TSM with glibenclamide was 0.6 ± 0.2.

The relaxant effects of different concentrations of *U. maritima* extract on incubated tissue with propranolol were significantly higher than the effect of saline ($p < 0.01$ for the second and $p < 0.001$ for higher extract concentrations). The relaxant effects of four higher concentrations of the extract in incubated TSM with propranolol were significantly lower than those in the nonincubated tissues ($p < 0.001$ for all cases, (Figure 3(a)). EC_{50} value of the *U. maritima* extract for its relaxant effect in incubated tissues with propranolol was significantly higher than that in the nonincubated TSM ($p < 0.001$) (Figure 2(a)). A rightward shift in concentration-response relaxation curve of the *U. maritima* extract was observed in propranolol-incubated TSM compared to nonincubated tissues but the maximum response was not achieved. The (CR-1) value of the extract in incubated TSM with propranolol was 2.3 ± 0.4.

Different concentrations of *U. maritima* extract caused significant relaxant effects in incubated tissues with diltiazem compared to the effect of saline ($p < 0.001$ for 5 last concentrations). The relaxant effects of 50 and 100 μg/ml of the extract in incubated tissue with diltiazem were significantly lower than those in the nonincubated TSM ($p < 0.05$ for both cases) (Figure 3(b)).

EC_{50} values of the *U. maritima* extract for its relaxant effect in incubated TSM with diltiazem were significantly higher than those in the nonincubated tissues ($p < 0.05$) (Figure 2(a)). A rightward shift in concentration-response relaxation curve of the extract was observed in diltiazem-incubated TSM compared to nonincubated tissues and the maximum response was achieved. The (CR-1) value of the extract in incubated TSM with diltiazem was 0.43 ± 0.1.

3.2. The Relaxant Effect of U. maritima Extract on TSM Contraction Induced by KCl in Nonincubated and Incubated

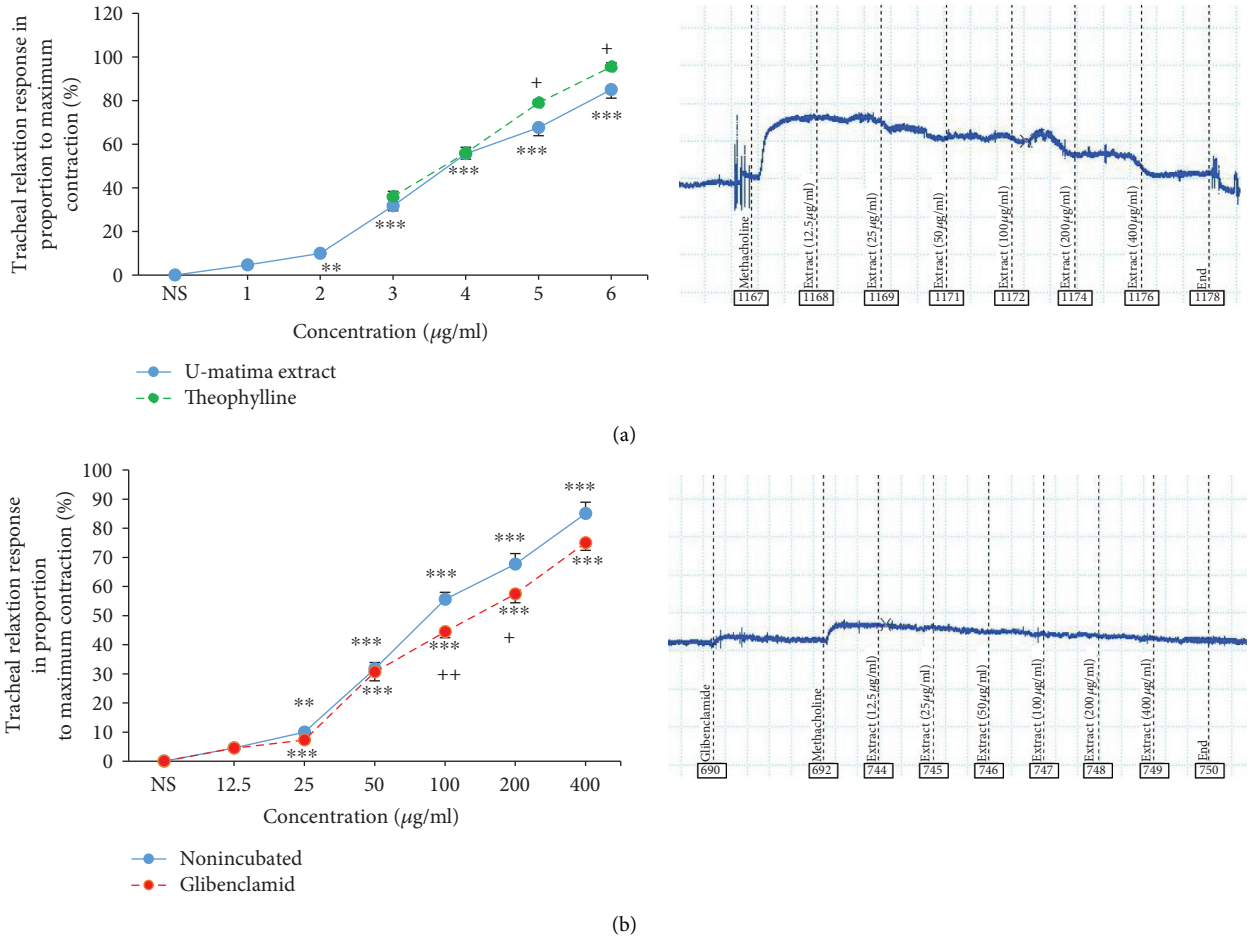

FIGURE 1: Concentration-response relaxant effects (mean ± SEM) of theophylline and *U. maritima* extract in nonincubated TSM contracted by 10 μM methacholine ($n = 7$). 1, 2, 3, 4, 5, and 6 in X-axis display six concentrations of the extract (12.5, 25, 50, 100, 20, and 400 μg/ml) and 3, 4, 5, and 6 display theophylline concentrations (0.2, 0.4, 0.6, and 0.8 mM) and (b) concentration-response relaxant effects (mean ± SEM) of theophylline and *U. maritima* extract in glibenclamide-incubated TSM (1 μM, $n = 8$). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the effect of saline (NS), $^{+}p < 0.05$ in panel (a) indicates comparison between the effect of theophylline and that of the extract. $^{+}p < 0.05$ and $^{++}p < 0.01$ in panel (b) show the comparison of the effect of the extract between incubated and nonincubated tissues. ANOVA with the Tukey–Kramer post hoc test was used for statistical comparison.

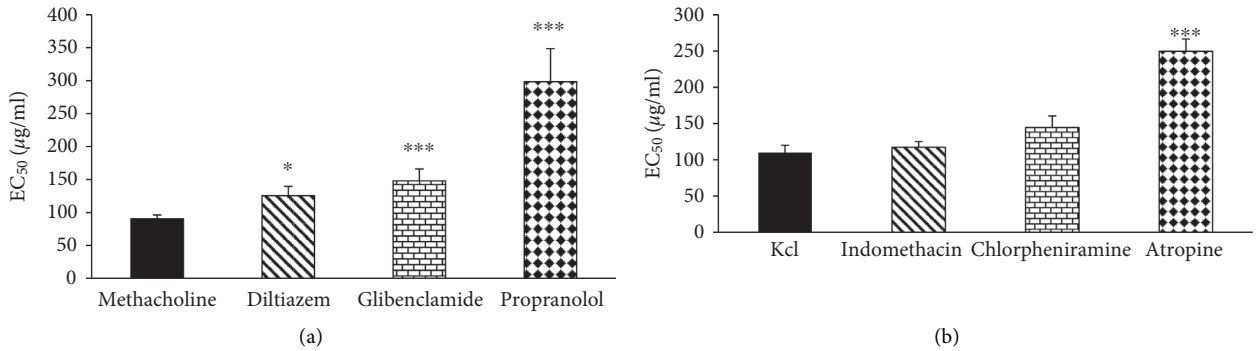

FIGURE 2: EC_{50} values of *U. maritima* extract-induced TSM relaxation in nonincubated and incubated TSM with various agents and contracted with methacholine (a) or KCl (b). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to nonincubated tissues. ANOVA with the Tukey–Kramer post hoc test was used for statistical comparison.

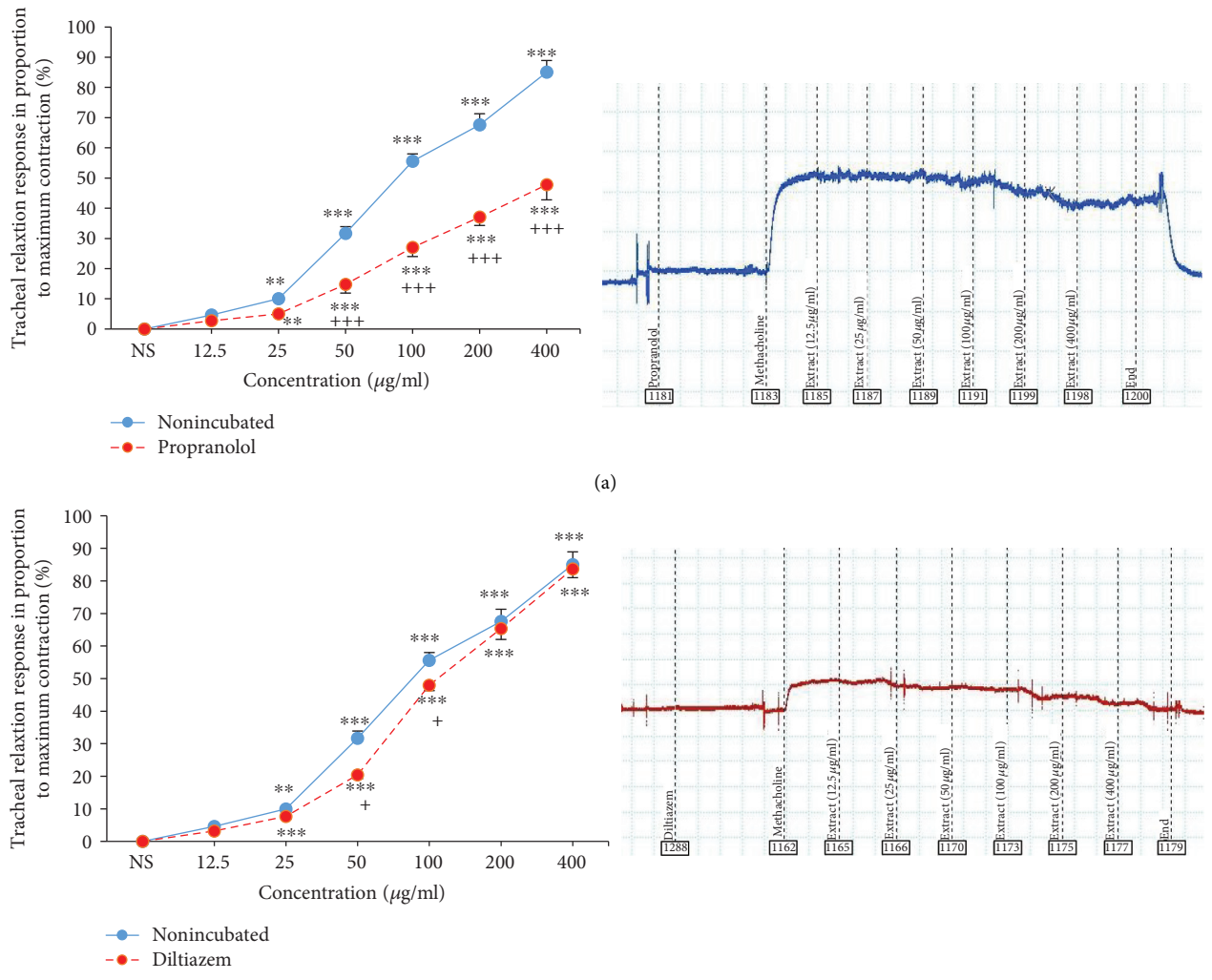

FIGURE 3: Concentration-response relaxant effects (mean ± SEM) of the *U. maritima* extract on 10 μM methacholine-induced contraction of TSM in nonincubated and propranolol-incubated (1 μM) (a) and diltiazem-incubated TSM (1 μM) (b) ($n = 8$ for all groups). $^{***}p < 0.001$, compared to the effect of saline (NS), $^{+}p < 0.05$, compared to the effect of the extract on nonincubated tissues. ANOVA with the Tukey–Kramer post hoc test was used for statistical comparison.

Tissues. In nonincubated TSM contracted by KCl, the relaxant effects of all concentrations of *U. maritima* extract and theophylline were higher than the effect of saline ($p < 0.001$ for all cases except the low extract concentration). The relaxant effects of 0.2, 0.6, and 0.8 mM theophylline were significantly higher than those of the corresponding concentrations of the extract ($p < 0.05$ to $p < 0.001$) (Figure 4(a)).

The extract of *U. maritima* showed significant and concentration-dependent relaxant effects on incubated TSM with atropine ($p < 0.05$ for 25 μg/ml and $p < 0.001$ for higher extract concentrations). The relaxant effects of four higher concentrations of the extract in incubated tissue with atropine were significantly lower compared to those in the nonincubated TSM ($p < 0.001$ for all cases) (Figure 4(b)).

EC_{50} values of the extract for its relaxant effect in incubated TSM with atropine were significantly higher compared to those in the nonincubated tissues with atropine ($p < 0.001$) (Figure 2(b)).

A rightward shift in concentration-response curve of the extract was seen in atropine-incubated TSM compared to nonincubated tissues and the maximum response was achieved. The (CR-1) value of the extract in incubated TSM with atropine was 1.6 ± 0.2.

The relaxant effects in 5 higher concentrations of extract in incubated TSM with chlorpheniramine and indomethacin were significantly higher compared to the effect of saline ($p < 0.05$ for 25 μg/ml in chlorpheniramine-incubated tissues and $p < 0.001$ for higher extract concentrations). There was no significant difference between the effects of different concentrations of extract in incubated TSM with chlorpheniramine and indomethacin and nonincubated tissues (Figures 5(a) and 5(b)).

3.3. Comparison of the Relaxant Effect of U. maritima Extract between TSM Contracted by Methacholine and KCl. There

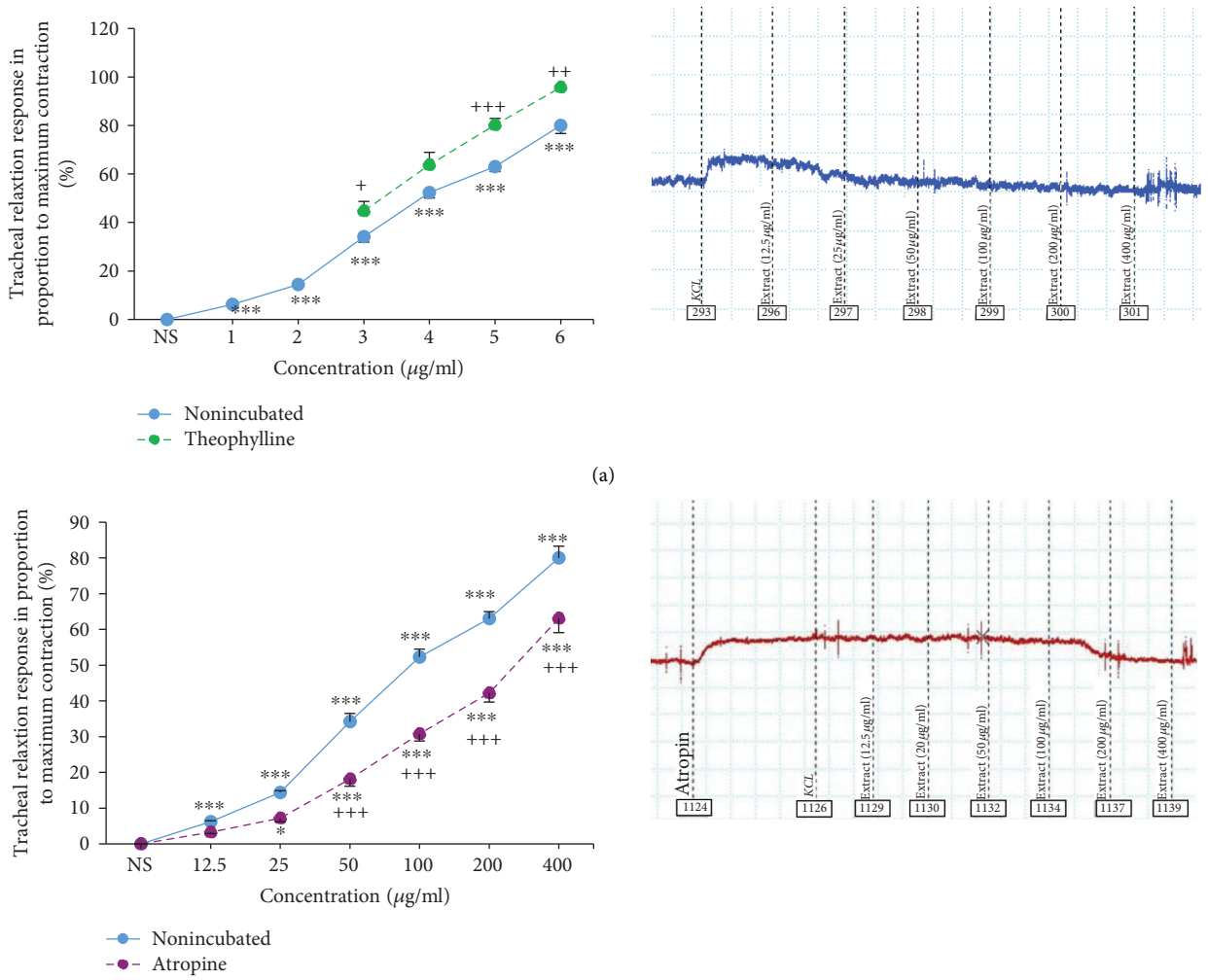

Figure 4: Concentration-response relaxant effect (mean ± SEM) of (a) theophylline and *U. maritima* extract in nonincubated TSM contracted by 60 mM KCl ($n = 8$). 1, 2, 3, 4, 5, and 6 in *X*-axis show six concentrations of the extract (12.5, 25, 50, 100, 20, and 400 μg/ml) and 3, 4, 5, and 6 show theophylline concentrations (0.2, 0.4, 0.6, and 0.8 mM) and (b) atropine-incubated TSM (1 μM, $n = 6$). $^{*}p < 0.05$ and $^{***}p < 0.001$, compared to the effect of saline (NS), $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$ in panel (a) indicate comparison between the effect of theophylline and that of the extract. $^{+++}p < 0.001$ in panel (b) shows the comparison of the effect of the extract between incubated and nonincubated tissues. ANOVA with the Tukey–Kramer post hoc test was used for statistical comparison.

was no significant difference between the relaxant effects of different concentrations of *U. maritima* extract between the TSM contracted by methacholine or KCl (Figure 6).

3.4. Correlations between Concentrations of the Extract of *U. maritima* and Theophylline with Their Relaxant Effects

The relaxant effects of theophylline and the extract were significantly correlated with their concentrations in all experimental groups ($p < 0.001$ for all cases) (Table 2).

4. Discussion

This study showed concentration-dependent relaxant effect of *U. maritima* extract in nonincubated TSM contracted by methacholine and KCl comparable to the effect of theophylline. The relaxant effect of *U. maritima* extract in nonincubated TSM contracted by methacholine and KCl was not significantly different. These results indicate a potent relaxant effect of the plant on TSM, which indicates its bronchodilatory effect in patients with obstructive pulmonary diseases. In fact, the effect of *U. maritima* in the treatment of respiratory diseases was indicated previously [23].

To examine the effect of *U. maritima* on β2-receptor [29], muscarinic [32], histamine (H1) [33] receptors, calcium channels [30], and potassium channels [34], ATP-sensitive potassium channels [34] and arachidonic acid metabolism [35] and their contribution in the relaxant effect of the plant were examined on tracheal smooth muscle incubated with propranolol, atropine, chlorpheniramine, diltiazem, glibenclamide, and indomethacin, respectively.

The relaxant effects of the extract in incubated tissues with propranolol, atropine, diltiazem, and glibenclamide

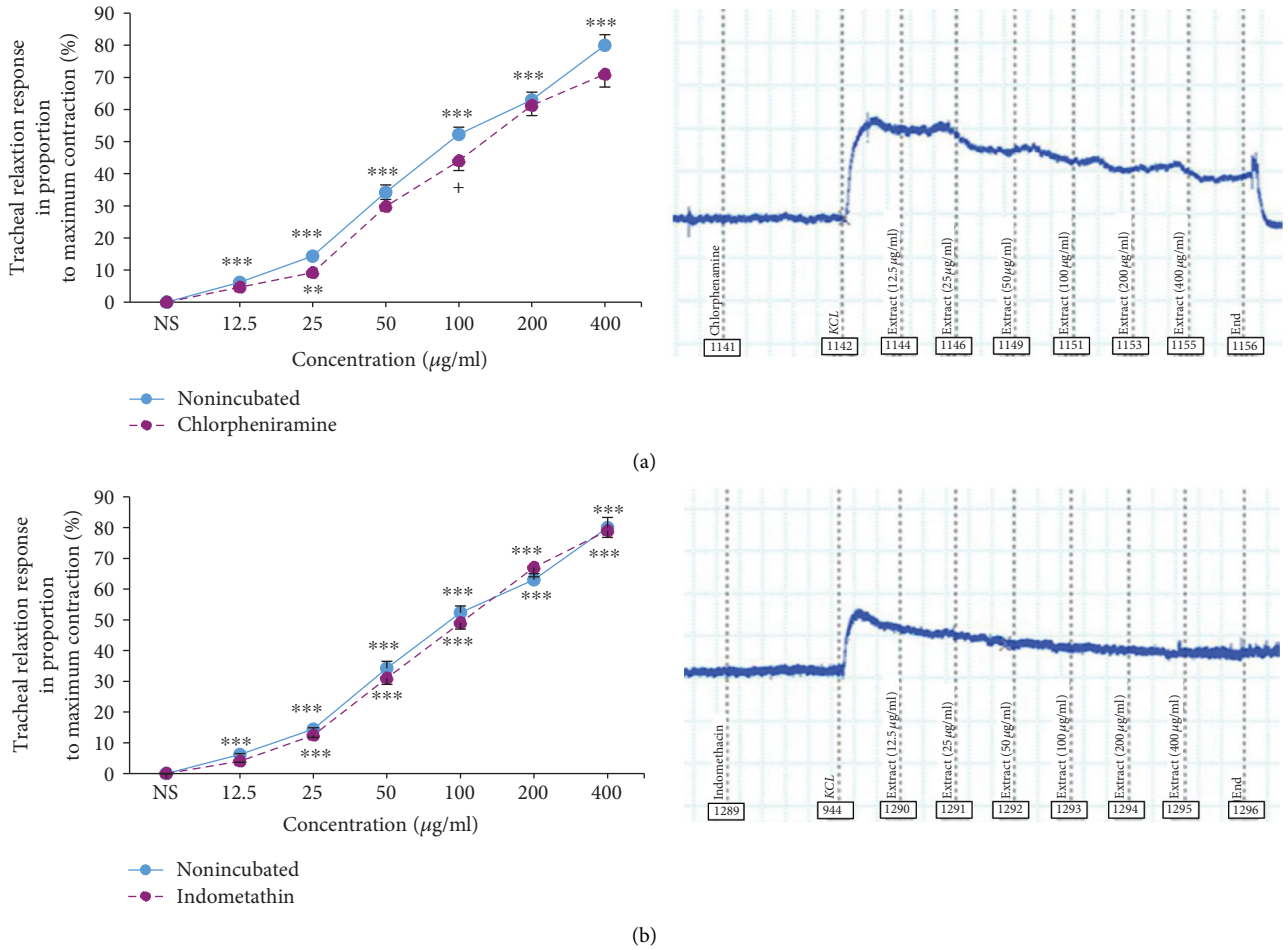

FIGURE 5: Concentration-response relaxant effects (mean ± SEM) of *U. maritima* extract on 60 mM KCl-induced contraction of TSM in nonincubated and chlorpheniramine-incubated (1 μM) (a) and indomethacin-incubated TSM (1 μM) (b) ($n = 8$ for all groups). $^{**}p < 0.01$ and $^{***}p < 0.001$, compared to the effect of saline (NS), $+p < 0.05$ compared to the effect of the extract on nonincubated tissues. ANOVA with the Tukey–Kramer post hoc test was used for statistical comparison.

were significantly lower than those in the nonincubated TSM. These results indicated the stimulatory effect of the plant on β2-adrenoceptor, inhibitory effect on muscarinic receptors, calcium channel blocking, and potassium channel opening effects, respectively. The EC_{50} values of the extract inducing relaxant effect in the incubated tissues with propranolol, atropine, diltiazem, and glibenclamide were also significantly higher compared to those in the nonincubated TSM. The higher EC_{50} values of the extract inducing relaxant effect in the incubated tissue also support the β2-adrenoceptor stimulation, muscarinic receptors inhibition, calcium channel blocking, and potassium channel opening properties of the plant. However, the maximum relaxant response was not obtained in incubated tissues with propranolol, which may indicate nonselective effect of the plant on β2-adrenoceptor [28]. The reason for the absence of maximum relaxant effect of the plant in incubated tissues with propranolol could be due to its effect on muscarinic receptors as well as calcium and potassium channels. The effect of another species of *U. maritima* extract on muscarinic receptors was demonstrated previously, which may support the effect of *U. maritima* on muscarinic receptor of TSM [24, 32]. Also, Memarzia and colleagues showed that the most important mechanism involved in relaxant effects of *Allium cepa* (*A. cepa*) extract was β2-adrenergic stimulatory and/or calcium channel [24], which may support the results of this study. A former study showed the relaxant activity of the extract of *Urginea indica* (another plant from Asparagaceae family) with the possible anticholinergic and Ca^{2+} antagonist mechanisms [27], which also supports the findings of the present study. However, the relaxant effects of *U. maritima* extract and its EC_{50} values in incubated tissues with chlorpheniramine and indomethacin were not significantly different from those in the nonincubated tissues. These results indicated the absence of the effect of the plant on histamine (H1) receptor [33] and arachidonic acid metabolism [35] pathways, ATP-sensitive potassium channels [34], and the contribution of these mechanisms to the relaxant effect of *U. maritima* extract on TSM.

This result showed relatively potent relaxant effect of *U. maritima* extract on TSM and the possible mechanisms of this effect for the first time. The mechanisms responsible for

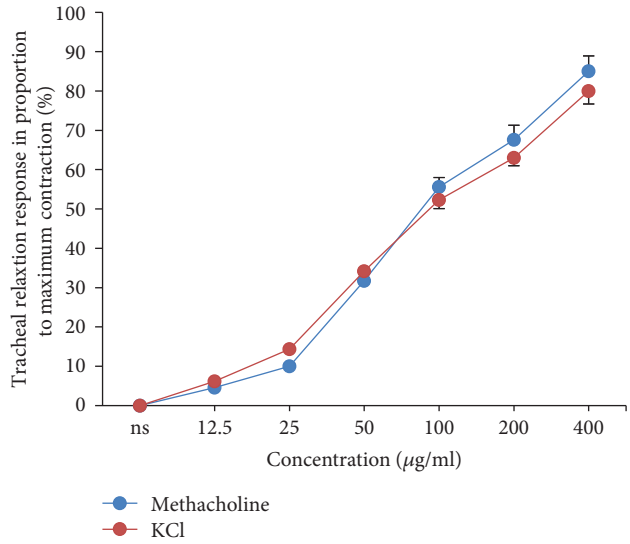

Figure 6: Concentration-response relaxant effects of the *U. maritima* extract in nonincubated tissues, contracted with 10 μM methacholine ($n = 8$) and 60 mM KCl ($n = 8$). There was no significant difference between the relaxant effects of the plant on methacholine or KCl-induced TSM contraction. ANOVA with the Tukey–Kramer post hoc test was used for statistical comparison.

Table 2: Correlations between different concentrations and the relaxant effects of *U. maritima* extract and theophylline.

Contractile agents	Studied agents	Conditions	R	p value
KCl	*U. maritime*	Nonincubated	0.967	$p < 0.001$
		Atropine-incubated	0.936	$p < 0.001$
		Indomethacin-incubated	0.976	$p < 0.001$
		Chlorpheniramine-incubated	0.965	$p < 0.001$
	Theophylline	Nonincubated	0.899	$p < 0.001$
		Nonincubated	0.960	$p < 0.001$
		Diltiazem-incubated	0.963	$p < 0.001$
Methacholine	—	Glibenclamide-incubated	0.961	$p < 0.001$
	U. maritima	Propranolol-incubated	0.946	$p < 0.001$
	Theophylline	Nonincubated	0.966	$p < 0.001$

Data were presented as mean ± SEM.

the relaxant effect of *U. maritima* extract on TSM are β2-adrenergic receptor stimulator, muscarinic receptors inhibition, calcium channel blocking, and potassium channels pathway opening effects or combinations of these mechanisms. The significant relaxant effect of *U. maritima* extract on TSM may indicate a bronchodilator effect for the *U. maritima* extract on obstructive pulmonary diseases.

5. Conclusions

In conclusion, this study displayed the potent relaxant effect of *U. maritima* on TSM comparable to the effect of theophylline, indicating its possible bronchodilatory property. Based on the results of this study, the possible mechanisms responsible for the relaxant effect of the plant on TSM are β2-adrenoceptor stimulation, muscarinic receptors inhibition, potassium channel opening, and calcium channel blocking properties.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgments

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Research Article

Thymoquinone Ameliorates Lung Inflammation and Pathological Changes Observed in Lipopolysaccharide-Induced Lung Injury

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Anti-inflammatory, antioxidant, and immunomodulatory effects of thymoquinone (TQ) have been shown. The effects of TQ on lipopolysaccharide- (LPS-) induced inflammation and pathological changes in rats' lung were investigated in this study. Four groups of rats included (1) control (saline treated); (2) LPS (treated with 1 mg/kg/day i.p. for two weeks); and (3 and 4) 5 or 10 mg/kg TQ i.p. 30 min prior to LPS administration. Total and differential WBC counts in the blood and bronchoalveolar fluid (BALF), TGF-β1, INF-γ, PGE2, and IL-4 levels in the BALF and pathological changes of the lung were evaluated. Total WBC count and eosinophil, neutrophil, and monocyte percentage were increased, but the lymphocyte percentage was reduced in the blood and BALF. The BALF levels of PGE2, TGF-β1, and INF-γ were also increased, but IL-4 level was reduced due to LPS administration. LPS also induced pathological insults in the lung of rats ($P < 0.05$ to $P < 0.001$ for all changes in LPS-exposed animals). Treatment with TQ showed a significant improvement in all changes induced by LPS ($P < 0.05$ to $P < 0.05$). TQ showed a protective effect on LPS-induced lung inflammation and pathological changes in rats which suggested a therapeutic potential for TQ on lung injury.

1. Introduction

Inflammatory response, under normal conditions, is a self-limiting process, but in several chronic diseases including lung injury, it could result in prolonged inflammation and tissue injury [1, 2]. It is well documented that overproduction of ROS in oxidative stress resulted in lung damage by various processes, including inflammation of the airways, which contribute to the pathogenesis and/or exacerbation of airways diseases [3] and the interrelationship between enhanced ROS generation and tissue inflammation [4].

Lung injury is commonly associated with endotoxemia, the presence of lipopolysaccharide (LPS) in the bloodstream; however, the mechanism that endotoxin induces the inflammatory response in acute lung injury is not well defined. Several studies reported inflammation in different organs induced by systemic administration of LPS, which is due to the production of reactive oxygen species (ROS) and proinflammatory cytokines [5, 6]. Our previous studies showed that intraperitoneal (i.p.) administration of LPS in the rats increased total and differential WBC count, induced oxidative damage by increasing the MDA level and

decreasing antioxidant markers, and led to a Th1/Th2 imbalance in the blood and the BALF [7–10]. Therefore, reducing oxidative injury and suppression of the inflammatory process could ameliorate LPS-induced tissue damage including lung injury.

In several animal models of lung injury, inflammation and oxidative stress are involved as the underlying pathophysiological mechanisms. Thus, anti-inflammatory or antioxidant agents have been widely used to alleviate lung injury. Thymoquinone (TQ), the main component of *Nigella sativa* (*N. sativa*) seeds, showed anti-inflammatory and antioxidant effects in various conditions including bronchial asthma.

Various studies indicated that administration of TQ ameliorated tracheal responsiveness to methacholine and ovalbumin, as well as total and differential WBC, especially eosinophilia [11], improved Th1/Th2 balance by enhancing the IL-4 but reducing IFN-γ levels, and improved pathological changes of the lung in the animal models of asthma [12–16]. Preventive and therapeutic effects of TQ in cyclophosphamide-induced lung injury in rats [17] and LPS-induced hepatotoxicity in mice [18] were also reported. In addition, TQ inhibited LPS-induced inflammatory mediators in BV2 microglial cells [19] and increased the expression of neuroprotective proteins and decreased the expression of proinflammatory cytokines and the gene expression of NFκB pathway signaling targets in LPS/IFNγ-activated BV-2 microglia cells [20]. TQ also suppressed production of Th2-type cytokines by mast cells in response to LPS stimulation in *vitro* [21].

Therefore, in this study, the effect of TQ on LPS-induced lung injury was examined. For this purpose, lung pathological changes, levels of cytokines in bronchoalveolar lavage fluid (BALF), and total and differential white blood cells (WBCs) counts in the BALF and blood of rats were evaluated in control, LPS-administrated, and LPS groups treated with TQ.

2. Materials and Methods

2.1. Animals and Drugs. Wistar rats (male < weighing 240 ± 10 g) were purchased from the animal house, Mashhad University of Medical Sciences, Mashhad, Iran, and kept in the same place in groups of 3 in individually ventilated cages with free access to food and water. Animals were maintained under temperature of $22 \pm 2^{\circ}$C, relative humidity of $54 \pm 2\%$, and 12 h light/dark cycles. The ethics committee of Mashhad University of Medical Sciences approved animal experimental protocols (Project ID 971117, 2 July 2017). Four groups of rats ($n = 6$ in each group) were studied as described in Table 1.

2.2. Blood and BALF Collection. The rats were sacrificed after deeply anesthetizing by 1.6 g/kg intraperitoneal (i.p.) administration of urethane at the end of the two weeks. The animals were sacrificed by a competent researcher with a minimum pain, suffering, and distress. The method was performed according the Annex IV of the guidelines from Directive EU/2010/63 of the European Parliament guideline. The blood samples were obtained by cardiac puncture. The blood samples were dispensed into the anticoagulant-containing tubes to be used for WBC count.

To prepare BALF samples, 1 ml phosphate buffered saline (PBS) was injected through a cannula inserted into the trachea of the right lung and was then aspirated. The procedure was repeated 5 times (total volume of 5 ml) [22, 23].

After sample collection, the heart of the rats was removed to euthanize the animals. The sample collection and euthanization of the animals were performed by a competent researcher with a minimum pain, suffering, and distress. The method was performed according the Annex IV of the guidelines from Directive EU/2010/63 of the European Parliament guideline [23, 24].

2.3. Blood and BALF Total and Differential WBC Counts. The cell pellets were suspended in normal saline after centrifuging BALF at 2500 rpm at 4°C for 10 min, and total and differential cell counts were measured.

Total WBC count was measured in 1 ml of blood or BALF stained with Turk's solution using a Neubauer counting chamber. Differential count of WBCs was determined in a smear of the blood or BALF stained with Wright–Giemsa, as described previously, under a light microscope [25].

2.4. Measurement of Cytokine Levels in the BALF. Interleukin-4 (IL-4), interferon-gamma (IFN-γ), transforming growth factor-beta-1 (TGF-β1), and prostaglandin-E2 (PGE2) levels in the BALF were measured by the specific ELISA kits, following the manufacturer's instructions (ebioscience Co., San Diego, CA, USA).

2.5. Pathological Evaluation of the Lung. The lung histological evaluation was performed as described previously (32). Briefly, the left lung was fixed in 10% buffered formalin (37%, Merck, Germany), and the lung specimens were embedded in paraffin, sectioned at 3-4 μm thickness, and stained with hematoxylin-eosin (H&E) solution. Using a light microscope and based on the following scoring system, inflammation, hemorrhage, interstitial fibrosis, epithelial damage, and emphysema changes were evaluated in the lug specimens of different groups. Pathological insult scores were defined as follows: 0, no pathological changes; 1, patchy changes; 2, local changes; and 3, severe changes (in most parts of the lung) [26].

2.6. Statistical Analysis. Comparison among groups was made using one-way analysis of variance (ANOVA) with Tukey multiple comparison tests. Data were presented as mean ± SEM. InStat (GraphPad Software, Inc., La Jolla, USA) software was used for statistical analysis, and $P < 0.05$ was used as statistical significance criteria.

3. Results

3.1. Total and Differential WBC Counts in the Blood and the BALF. LPS administration resulted in significantly increased total WBC count and eosinophil, neutrophil, and

Table 1: Studied groups, lipopolysaccharide (LPS) administration and thymoquinone (TQ) treatment.

Group	Saline or LPS administration	TQ treatment	Abbreviation
Control	Saline, 2 ml/kg/day, i.p. for two weeks	Saline, 30 min before LPS administration, i.p.	C
LPS administrated	LPS, 1 mg/kg/day, i.p. for two weeks	"	LPS
LPS + TQ 5 mg/kg	"	TQ, 30 min before LPS administration, i.p.	LPS-TQ 5
LPS + TQ 10 mg/kg	"	"	LPS-TQ 10

LPS and TQ were purchased from Sigma Chemical Co. LPS was dissolved in warm sterile saline supplemented with ethanol, and TQ was dissolved in cold sterile saline both freshly prior to injection. The doses of TQ [17, 18] and LPS [12–15] were chosen according previous studies.

monocyte percentages of in the both the blood and the BALF but a significant decrease in the lymphocyte percentage compared to the control group ($P < 0.05$ to $P < 0.001$). In the blood, total WBC count and percentages of neutrophil and monocyte were decreased, but lymphocyte percentage in the both blood and BALF was increased in treated groups with both doses of TQ. However, eosinophil percentage in the blood as well as total WBC count and percentages of eosinophil, neutrophils, and monocytes in the BALF were reduced and in rats treated with the higher dose of TQ compared to the LPS group ($P < 0.05$ to $P < 0.001$) (Figures 1–4).

3.2. Cytokine Levels in the BALF. Levels of PGE2, TGF-β1, and INF-γ were significantly enhanced, but IL-4 was significantly reduced in the BALF of the LPS-administered group as compared to the control rats ($P < 0.001$ for all cases). Treatment with both doses of TQ significantly reduced PGE2, TGF-β1, and INF-γ but increased IL-4 levels compared to the LPS group ($P < 0.001$ for all cases) (Figures 5 and 6).

3.3. Lung Pathology. In the LPS group, pathological insults in the lung including fibrosis, interstitial inflammation, hemorrhage, epithelial damage, and emphysema were significantly increased ($P < 0.05$ to $P < 0.001$). Treatment with both TQ doses significantly ameliorated all pathological changes of the lung induced by LPS ($P < 0.05$ to $P < 0.001$) (Figures 7–9).

4. Discussion

The effect of TQ on lung injury induced by LPS in rats and the possible mechanisms underlying this effect were examined in this study. Previously, the anti-inflammatory effect of LPS was shown in the lung of animal models [27]. In this study, LPS injection for two weeks increased total and differential WBC counts in the BALF and the blood. Furthermore, LPS decreased the IL-4 level but increased IFN-γ, TGF-β1, and PGE2 levels in the BALF. Moreover, pathological insults of the lung such as lung inflammation, epithelial damage, emphysema, and fibrosis confirmed LPS-induced lung injury in the current study which was also shown in the previous studies [8, 10].

Based on the results of previous studies, chronic inflammatory processes in the lung were induced by long-term exposure to LPS [28]. Most studies showed that neutrophil infiltration into the lung tissue is an important inflammatory response factor of the lung to LPS administration [29]. In fact, bronchoalveolar neutrophilia was reported as the main cell response following LPS inhalation [30]. In the present study, increased total WBC count and percentages of eosinophils, neutrophils, and monocytes in the BALF and the blood showed systemic and lung inflammation induction. In the LPS-administered animals, decreased lymphocytes percentage was due to increased total WBC count. In fact, increased absolute lymphocytes count was demonstrated in previous studies [8, 10].

A previous study also showed systemic inflammation caused by the release of inflammatory cytokines following i.p. injection of LPS [8]. It was shown that LPS induced Th1 responses (IFN-γ) and inhibited Th2 responses (IL-4) through the toll-like receptor 4- (TLR4-) dependent pathway; also, LPS increased BALF levels of PGE2 and TGF-β1 [8]. TLR4 is the main receptor for LPS. The expression of TLR4 on mononuclear cells may be related to the exogenous LPS level and affects the balance of Th1/Th2 cells in the opposite way to that of LPS alone [27]. Therefore, the results of the current study in terms of systemic and lung inflammation due to chronic LPS administration are supported by the abovedescribed studies.

The emphysematous changes in lung architecture could occur due to chronic LPS exposure which can result from neutrophil infiltration, chronic inflammatory responses, airway wall thickening, mucus cell metaplasia, and irreversible alveolar enlargement [28, 31]. It is accepted that lung inflammation is the main characteristic of lung injury [8]. Pathological findings of the lung in the present study demonstrated that LPS induced lung injury, similar to the abovenoted studies.

The results of the present study showed a protective effect of TQ on LPS-induced lung inflammation dose dependently. Application of TQ decreased total and differential WBC counts in the blood and inhibited neutrophil, eosinophil, and monocyte infiltration into the airways when coadministered with LPS which indicated the improvement of systemic and lung inflammation. Administration of TQ increased Th2 response (IL-4), inhibited Th1 response (IFN-γ), and resulted in the improvement in Th1/Th2 immune homeostasis. Pretreatment with TQ caused considerable improvement of lung inflammation by decreased BALF levels of PGE2 and TGF-β1 as well as ameliorated pathological changes in the lung which all confirmed the protective effects of TQ on lung inflammation. However, the percentage of basophiles was very little to present their results in all studied groups both in the BALF and the blood. In addition, it was much more reliable if total and differential

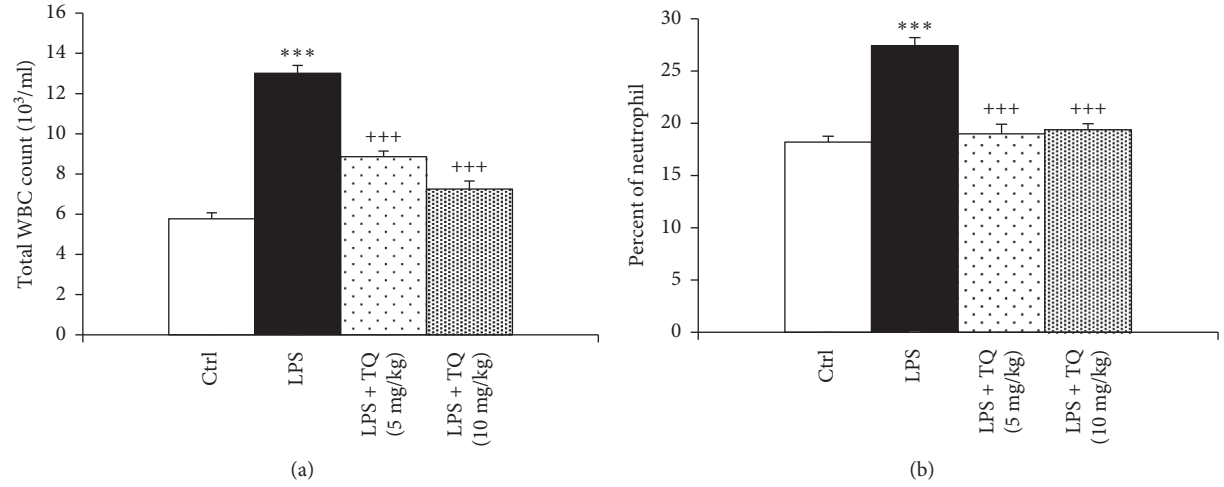

FIGURE 1: Total white blood cell count (a) and percent of neutrophils (b) in blood. Data are presented as means ± SEM ($n = 6$ in each group). $^{***}P < 0.001$ vs. control group; $^{+++}P < 0.001$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with Tukey multiple comparison tests was used.

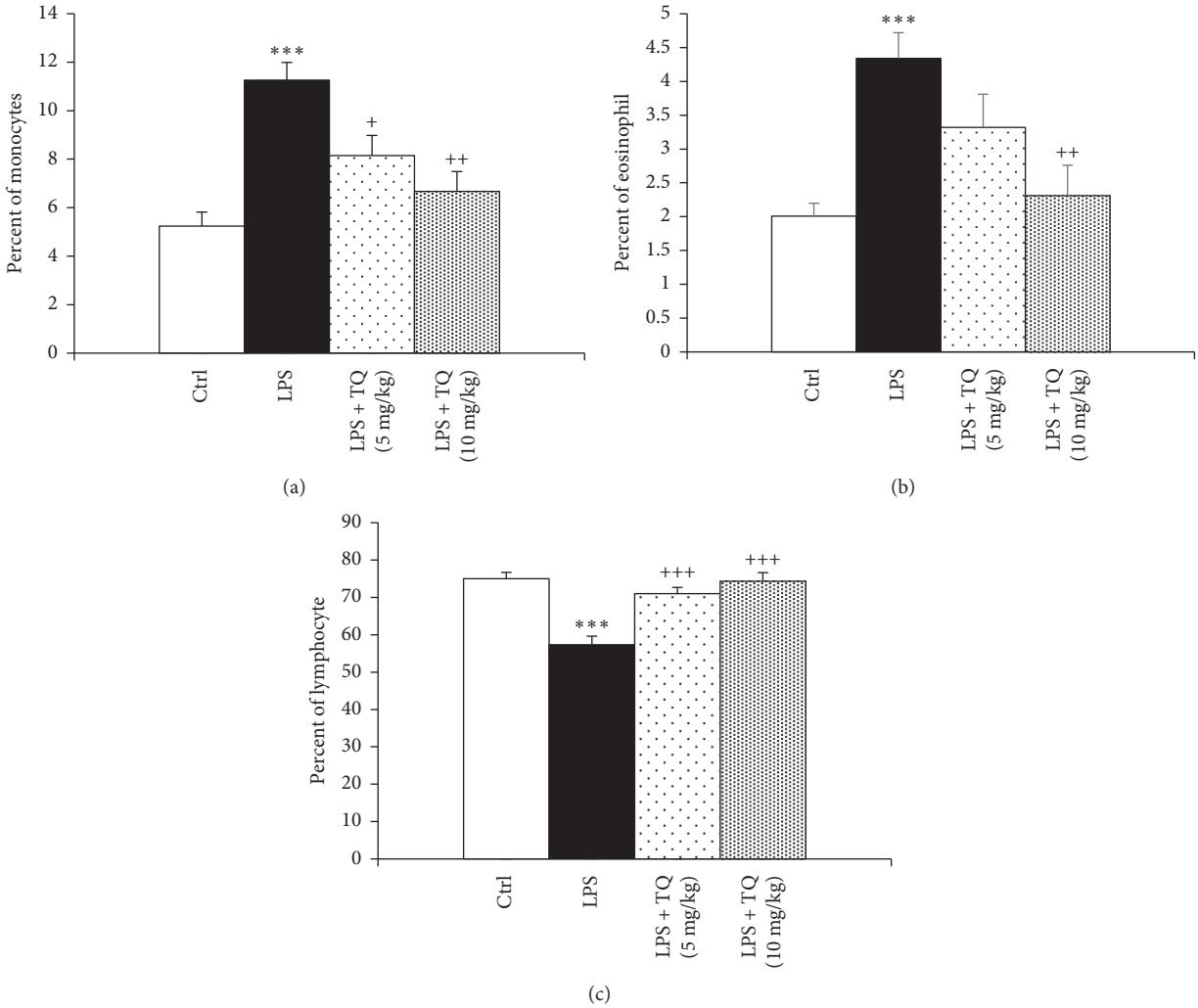

FIGURE 2: Percentages of monocyte (a), eosinophil (b), and lymphocytes (c) in blood. Data are presented as means ± SEM ($n = 6$ in each group). $^{***}P < 0.001$ vs. control group; $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with tukey multiple comparison tests was used.

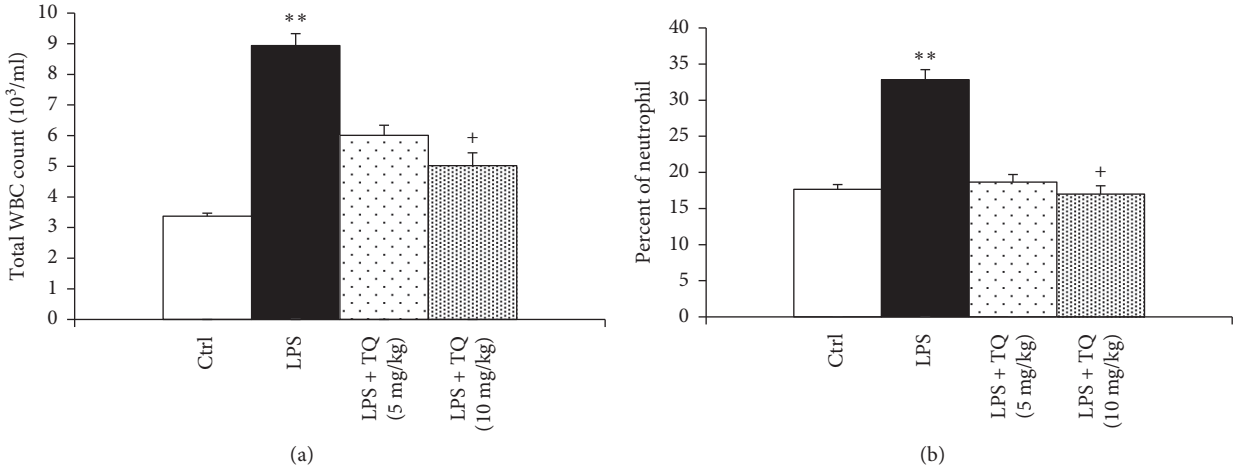

FIGURE 3: Total white blood cell count (a) and percent of neutrophils (b) in the BALF. Data are presented as means ± SEM ($n = 6$ in each group). $^{**}P < 0.01$ vs. control group; $^{+}P < 0.05$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with tukey multiple comparison tests was used.

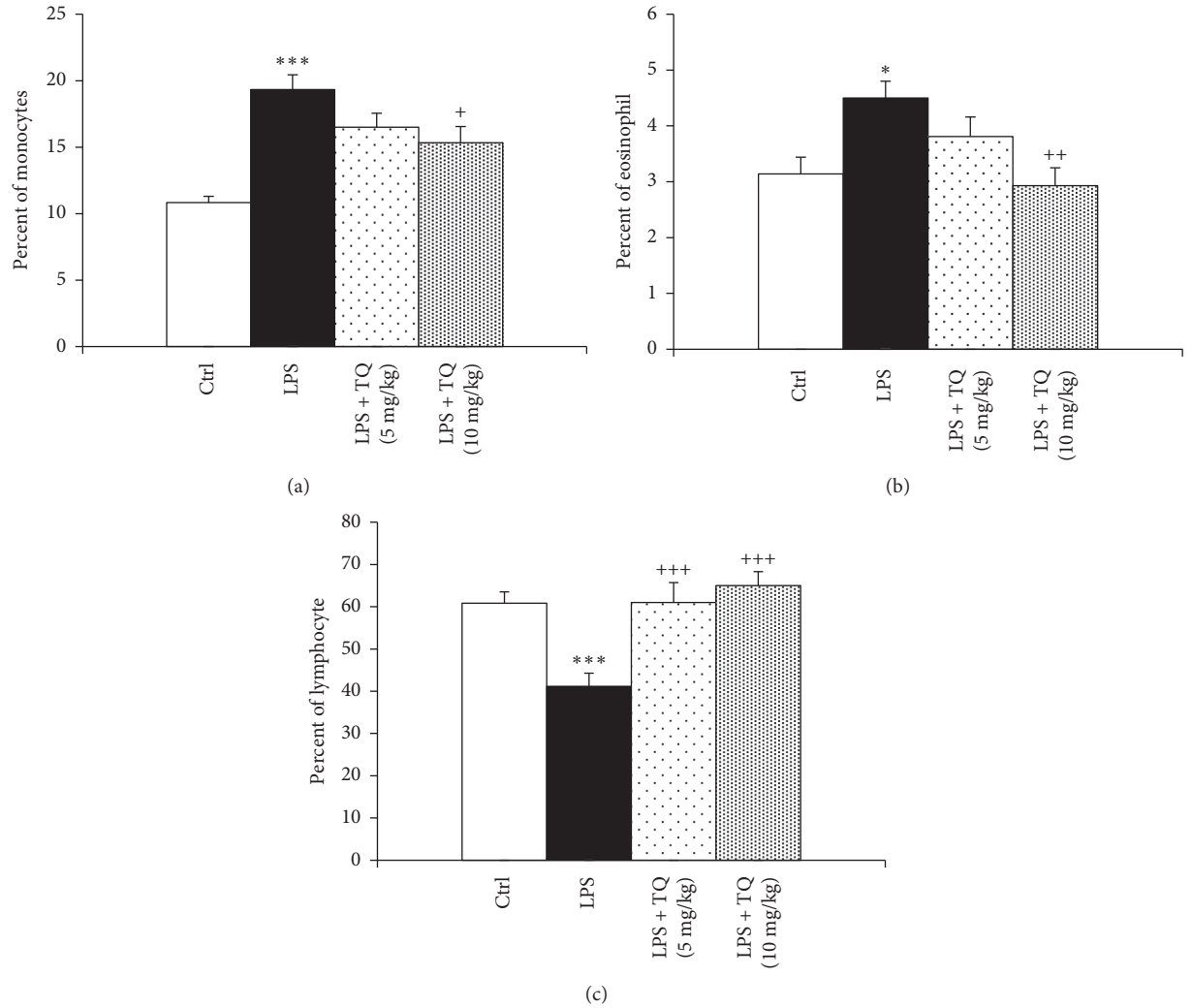

FIGURE 4: Percentages of monocyte (a), eosinophil (b), and lymphocytes (c) in the BALF. Data are presented as means ± SEM ($n = 6$ in each group). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control group, and $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with tukey multiple comparison tests was used.

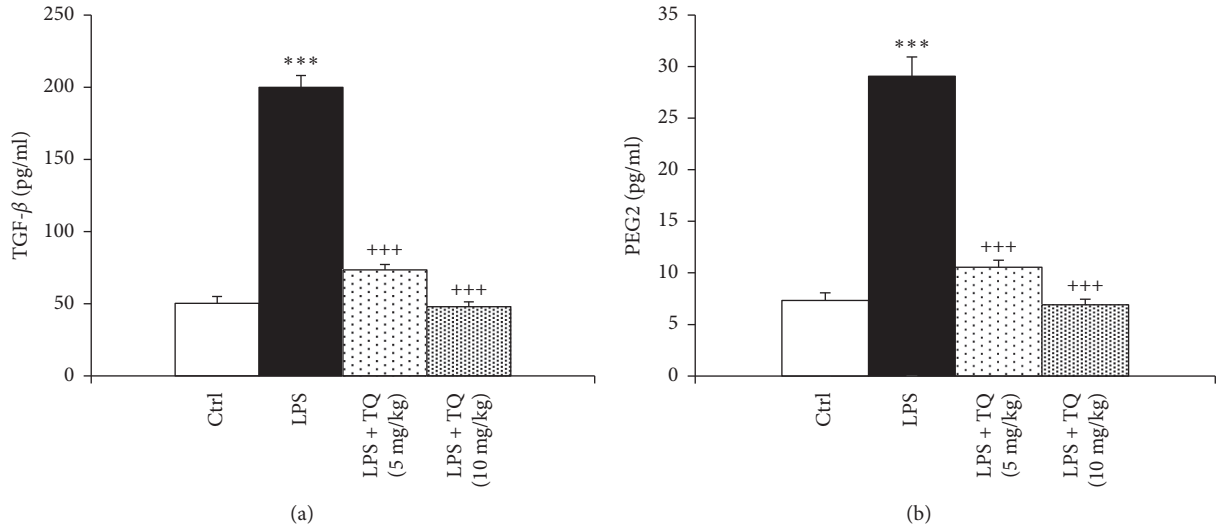

Figure 5: The concentrations of TGF-β1 (a) and PGE2 (b) in the BALF. Data are presented as means ± SEM ($n = 6$ in each group). $^{***}P < 0.001$ vs. control group; $^{+++}P < 0.001$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with Tukey multiple comparison tests was used.

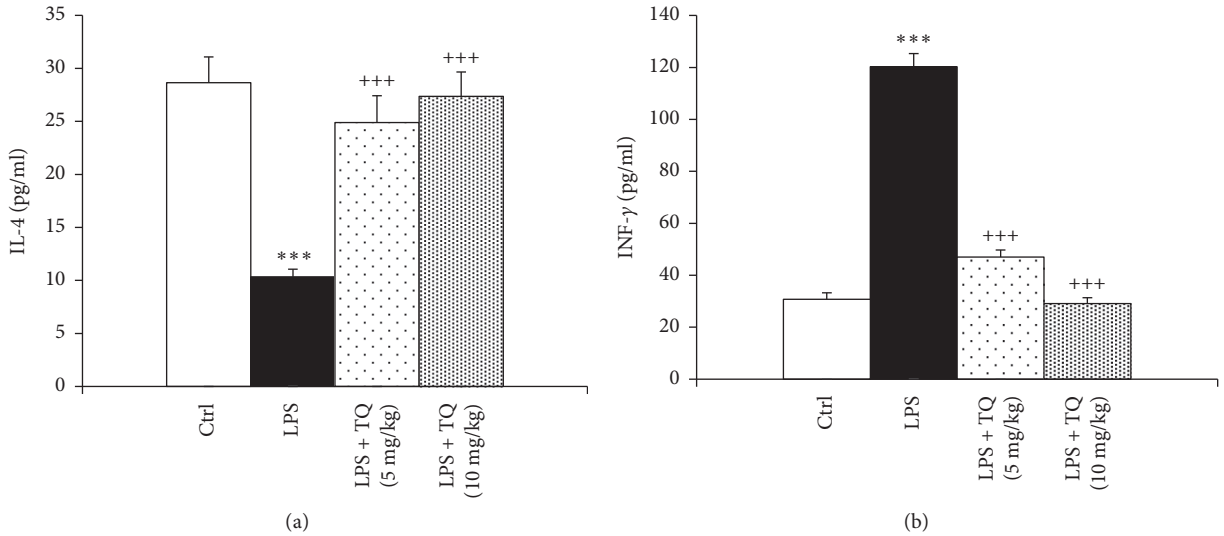

Figure 6: The concentrations of IL-4 (a) and INF-γ (b) in the tissue. Data are presented as means ± SEM ($n = 6$ in each group). $^{***}P < 0.001$ vs. control group; $^{+++}P < 0.001$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with tukey multiple comparison tests was used.

WBC counts in the blood and BALF were evaluated by flow cytometry, but their evaluation by the traditional method is still acceptable way and it was tried hard to avoid human errors in counting total and differential WBC.

The anti-inflammatory effect of TQ on lung inflammation in animal models of asthma was previously reported [12, 14–16, 32]. Evidence suggests that TQ decreases airway inflammation by stimulating IFN-γ secretion (Th1-mediated cytokine), inhibiting IL-4, IL-5, and IL-13 secretion (Th2-mediated cytokines), NF-κB, IL1β, TNFα, and cyclooxygenase (COX)-1 expression, and PGE2 and PGD2 production, leukotriene (LT) B4 and LTC4 synthesis and eosinophil infiltration into the airways [15, 16, 32–34]. Furthermore, histopathological examinations, in previous reports, confirmed the anti-fibrotic effect of TQ [35, 36]. The ameliorative effect of TQ, mediated via cytokines rearrangement, was also indicated on LPS-induced pulmonary blood vascular damage in rats [37].

In this study, TQ not only reduced infiltration of WBCs, especially neutrophils, as one of the main component of the inflammatory response, into the lung but also decreased total and differential WBC counts in the blood and, thereby, reduced systemic inflammation.

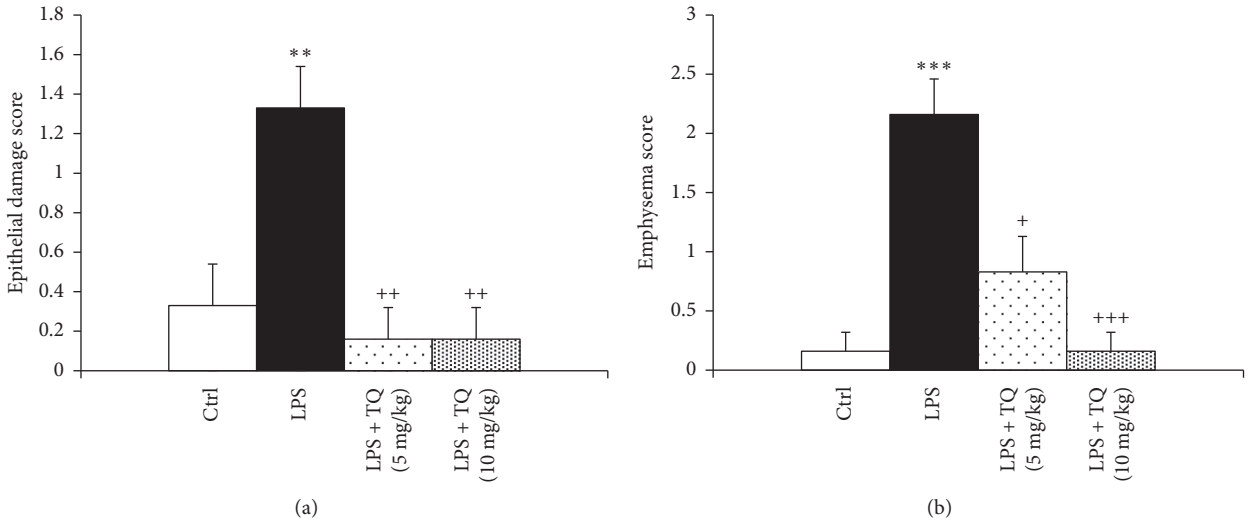

Figure 7: Epithelial damage (a) and emphysema (b) scores of lung tissue. Data are presented as means ± SEM ($n = 6$ in each group). $^{**}P < 0.01$ vs. control group; $^{++}P < 0.01$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with tukey multiple comparison tests was used.

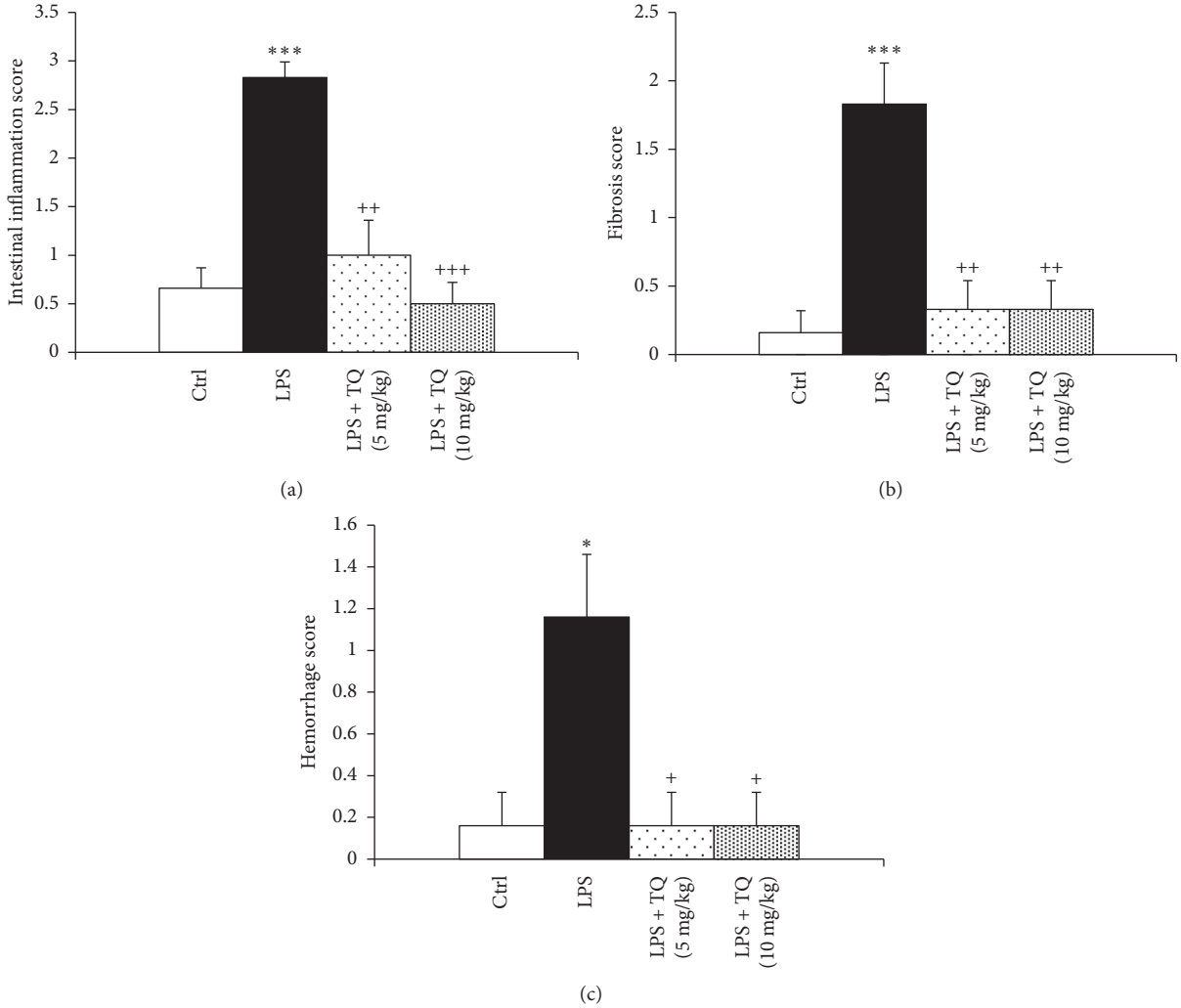

Figure 8: Intestinal inflammation (a), fibrosis (b), and hemorrhage (c) scores of lung tissue. Data are presented as means ± SEM ($n = 6$ in each group). $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. control group; $^{++}P < 0.01$ and $^{+++}P < 0.001$ vs. LPS group. For comparison between groups, one-way analysis of variance (ANOVA) with Tukey multiple comparison tests was used.

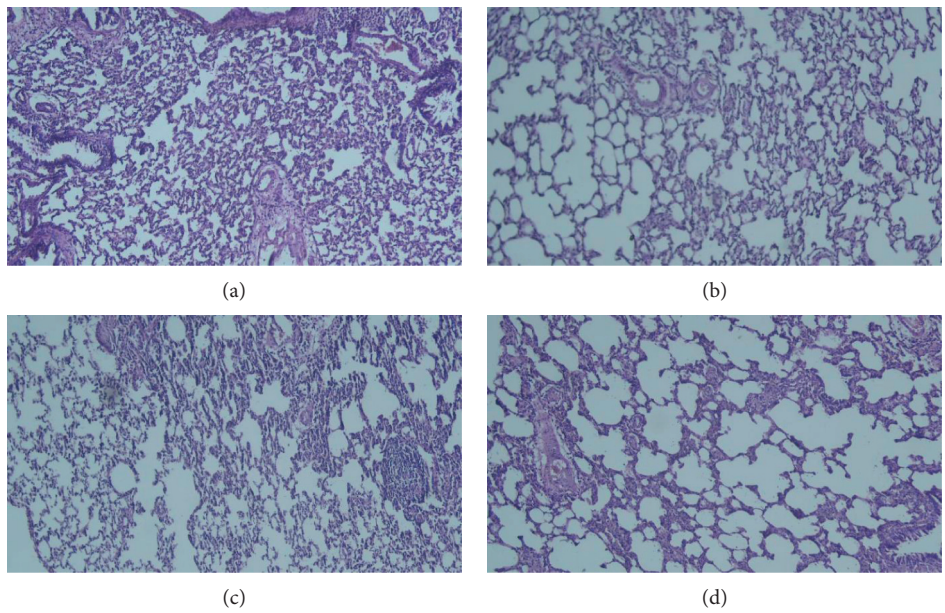

Figure 9: Representative photographs of pathological changes of lung in LPS-induced lung in the control group (a), LPS-exposed group (b), and LPS-exposed and -treated animals with low (c) and high (d) doses of TQ (hematoxylin and eosin staining, magnification 200x).

The exact mechanisms of the anti-inflammatory activity of TQ in LPS-induced lung injury are not clear. TQ was shown to inhibit eicosanoid production and reduce proinflammatory lipid mediators in *vitro*. COX-1 is a constitutively expressed enzyme with various physiological functions. TQ was shown to inhibit the synthesis of PGE2 by COX-1 and COX-2, in *vitro* [32]. This study suggests that the anti-inflammatory activity of TQ is probably mediated by a mechanism that targets COX-2-mediated PGE2 synthesis.

This study showed that TQ inhibited Th1 cell-mediated response and IFN-γ production as well as shifted the immune response toward a Th2-dominant pattern and IL-4 production. In this study, TQ-induced IL-4 production and Th2 cell-mediated response which probably play a key role in damping inflammation and LPS-induced lung injury.

Three isoforms of TGF-β as a master switch in the fibrotic process including TGF-β1, TGF-β2, and TGF-β3 existed in mammals. TGF-β1 is the isoform which is most closely related to the development of idiopathic pulmonary fibrosis [38]. It was indicated that TGF-β overexpression and TGF-β-smad3 signaling are implicated in pulmonary fibrosis and emphysema in experimental rodent models [39]. In the current study, TQ reduced TGF-β1 level and prevented the development of lung fibrosis and emphysema in LPS-induced lung injury. In fact, the effects of TQ in pulmonary vascular damage induced by LPS via cytokine downregulation in rats [37] and its effect on activated BV-2 microglial cells by LPS via reduction of NO2 and iNOS protein expression and improvement in various cytokines such G-CSF, MCP-5, MCP-1, and IL-6 protein in activated BV-2 cells [40] supporting the results of the current study were reported. The inhibitory effects of TQ on IL-4 production, OVA-specific IgE, TNF-α and IL-1b gene expression, edema, and eosinophil infiltration in the nasal mucosa were reported in a rat model of allergic rhinitis [41]. Antinociceptive and anti-inflammatory effects of TQ such as radical scavenging activity and interaction with proinflammatory enzymes and cytokines were indicated [42]. The effect of TQ on biosynthesis of various inflammatory mediators (5-LO, COX, PGD2, and LTs), reducing proinflammatory cytokines (interleukins (ILs) and TNF-α), reducing oxidative stress, and increasing chemokinesis, chemotaxis, phagocytic activity, antibody levels, and the hemagglutination, was also shown [43].

In this study, administration of TQ before LPS-induced inflammation showed apreventive effect on the development of lung inflammation. Therefore, it remains to be investigated whether TQ has a similar anti-inflammatory activity in the presence or after the induction of lung inflammation. Although a large number of studies have been published on anticancer and anti-inflammatory effect of TQ in the last 5 years, this study examined the effect of TQ in LPS-induced (i.p. administred) lung injury with different processes compared to previous studies for the first time.

Total and differential WBC in the blood as an indicator of systemic inflammation as well as total and differential WBC in BALF, BALF levels of various cytokine, and lung pathological changes as indicators of lung injury were measured in this study. However, in further studies, more precise mechanisms of LPS-induced lung injury and the protective effect of TQ including more inflammatory and oxidative stress markers should be evaluated.

5. Conclusions

The preventive effect of TQ on LPS-induced inflammation and lung injury was shown by reducing total and differential WBC airway inflammatory cells, lung hemorrhage,

interstitial inflammation, epithelial damage and emphysema, fibrosis, and balancing Th1/Th2 immune response.

Data Availability

The data are available on request from the corresponding author Mohammad Hossein Boskabady (boskabadymh@mums.ac.ir).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Anti-Inflammatory Effects of *Sosiho*-Tang, a Traditional Herbal Formula, on Acute Lung Injury in LPS-Sensitized Mice and -Raw 264.7 Cells

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Acute lung injury (ALI) is a series of syndromes with persistent inflammation and abnormally increased vascular permeability. *Sosiho*-tang (SSHT), a traditional herbal formula consisting of a mixture of seven herbs, has been used to treat allergic reactions and chronic hepatitis disease in East Asia. In this study, we determined whether SSHT has an inhibitory effect against lipopolysaccharide- (LPS-) induced acute lung injury (ALI) in mice. 0.05, 0.55, and 5.55 mg/kg of SSHT were orally administered to C57BL/6J mice for 7 days prior to the administration of LPS. After 2 h of LPS sensitization, lung tissues were collected to confirm the lung histology and ALI-related inflammatory factors. SSHT ameliorated the LPS-induced alveolar hemorrhage, alveolar wall thickening, and the shrinkage of the alveolar spaces in the ALI mice model. Proinflammatory cytokines including IL-6, TNF-α, and IFN-γ in the lung tissue were significantly regulated in the SSHT-treated groups compared to the LPS only-treated group. Also, increases of IL-6 and TNF-α and decrease of IFN-γ expressions were dose-dependently modulated by SSHT treatment in LPS-induced raw 264.7 cells. Additionally, the translocation of NF-κB into nucleus and phosphorylation of mitogen-activated protein (MAP) kinase were significantly attenuated by the treatment of SSHT in LPS-sensitized ALI mice. SSHT showed anti-inflammatory activities by inhibiting proinflammatory cytokines and NF-κB signaling in LPS-induced ALI. This study demonstrates that SSHT has preventive effects on LPS-induced ALI by regulating inflammatory responses as an alternative for treating lung diseases.

1. Introduction

Acute lung injury (ALI) is a life-threatening medical complication with a range from 52 to 65% of mortality rate in patients [1]. ALI is associated with sepsis, traumatic injuries, inhalation, massive blood transfusion, bilateral pulmonary infiltration, and hypoxemia [2]. It is commonly accompanied by systemic inflammatory responses following pulmonary edema, increase of alveolar capillary barrier permeability, and excessive cytokines production in lung [3–5]. In the previous studies, lipopolysaccharide (LPS), component of Gram-negative bacteria, has been known to induce the release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which stimulates alveolar hemorrhage, alveolar wall thickening, and alveolar spaces shrinkage [6, 7].

Clinically, treatment of ALI focuses on counterbalance of inflammatory response by regulating inflammatory/anti-inflammatory cytokines production [8]. Some studies have reported that methylprednisolone reduces ventilation, respiratory shock, and mortality rate in ALI patients by improving the systemic inflammatory response [9]. However, the efficacy of methylprednisolone, one of the representative corticosteroids, is controversial, because some studies reported no significant benefit on ALI in clinical trials [10, 11]. In addition, there were unexpected side effects of corticosteroids such as hyperglycemia, hypertension, and further severe neuronal disorder [12, 13]. Commonly, inhalation of

oxygen and restriction of intravenously received fluids are primary treatments for ALI; however, they may lead to pulmonary edema and interstitial pneumonitis due to the increase of pulmonary venous oxygenation caused by hyperoxia [14, 15]. Novel treatment derived from natural products with no side effects is required to treat ALI.

Sosiho-tang, known as Sho-saiko-to in Japanese and Xiao-Chai-Hu-Tang in Chinese, is an herbal medicine in Korea for medical insurance. *Sosiho*-tang consists of seven herbs: *Bupleurum falcatum* Linne 12 g, *Scutellaria baicalensis* Georgi 8 g, *Pinellia ternata* Breitenbach 4 g, *Panax ginseng* C. A. Meyer 4 g, *Glycyrrhiza glabra* Linn var. *glandulifera* Regel & Herder 2 g, 3 of *Zingiber officinale* Roscoe, and 2 of *Ziziphus vulgaris* Lamarck var. *inermis* Bunge. It is widely used for treating allergic diseases, cold-related symptoms, and chronic liver disease [16–19]. In particular, diseases for which medicine is efficacious of *Sosiho*-tang include the dyshepatia and hepatosis of chronic liver disorders [20, 21]. In the preclinical studies, *Sosiho*-tang improved liver inflammation and fibrosis by regulating liver toxicity levels, demonstrating that research of *Sosiho*-tang is focused on liver disease [19, 22, 23]. Because *Sosiho*-tang is reported to decrease the production of T helper 2-type cytokines and chemokine [24], we postulated that *Sosiho*-tang has a therapeutic effect on ALI via its anti-inflammatory effects. In the present study, inflammatory effects of *Sosiho*-tang against ALI were determined in LPS-sensitized mice and raw 264.7 cells.

2. Materials and Methods

2.1. Sosiho-Tang Soft Extract Sample. The sample of *Sosiho*-tang used in this study was Jungwoo Pharmacy *Sosiho*-tang Soft Ext. (SSHT), a prescription-based medicine in Korea. SSHT, a Korean Medicine for national health insurance, was obtained from Jungwoo Pharmacy Co., Ltd. (Lot. no. 702). Briefly, SSHT consists of 750 mg of *Bupleurum falcatum* Linne, 1.115 g of *Scutellaria baicalensis* Georgi, 588 mg of *Pinellia ternate* Breitenbach, 514 mg of *Panax ginseng* C. A. Meyer, 246 mg of *Glycyrrhiza glabra* Linn var. *glandulifera* Regel & Herder, 75 mg of *Zingiber officinale* Roscoe, and 518 mg of *Ziziphus vulgaris* Lamarck var. *inermis* Bunge soft extracts based on the general requirements in the Korean Pharmacopoeia. A total of 9 g of SSHT was dissolved in distilled water with appropriate doses for mice.

2.2. Animal Treatment. Thirty-six males C57BL/6 mice at 5 weeks of age were purchased from Raon Bio Inc. (Yongin, Korea). Mice were housed under controlled conditions (at 24 ± 2°C, relative humidity of 50–80%, and 12 h light/dark cycle). After 1-week acclimation, mice were randomly divided into six groups with 6 per group: CTR (nontreated group as a normal control), LPS (LPS-sensitized group as a negative control), DEX (LPS-sensitized and DEX-treated group as a positive control), S0.05 (LPS-sensitized and SSHT 0.05 g/kg-treated group), S0.55 (LPS-sensitized and SSHT 0.55 g/kg-treated group), and S5.55 (LPS-sensitized and SSHT 5.55 g/kg-treated group). 7 mg/kg of dexamethasone (DEX) and SSHT were orally preadministrated for 1 week, followed by intraperitoneal injection of 10 mg/kg LPS. The SSHT that was used for the human being 9 g/60 kg/day was converted by using the human equivalent dose (HED) equation used for mice into 5.55 g/kg. There was no toxicity to mice during sample administration of SSHT 5.55 g/kg. Following LPS sensitization for 2 h, blood samples were collected by cardiac puncture. Lung tissues were collected at 10% neutralized formalin solution. All experiments were approved by the Committee on Care and Use of Laboratory Animals of Kyung Hee University (KHUASP(SE)-18-078; Seoul, Korea).

2.3. Histological Analysis. The right inferior lobe of lung was fixed at 10% neutralized formalin for 24 h. The lung tissues were dehydrated and embedded in paraffin for 24 h. The paraffin blocks were cross cut into 4 μm thickness and mounted on slides. These slides were stained with hematoxylin and eosin solution. The stained slides were photographed by digital microscope at a magnification of 200× and 400×. The blind test for ALI score was determined as 0 to 5 grades: 0, no injury and appears normal; 1, minimal (injury up to 25% of the field); 2, mild (injury between 25 and 50% of the field); 3, moderate (injury between 50 and 75% of the field); and 4, severe (>75%, diffuse injury). Tissue sections were examined by a pathologist blinded to the experiment.

2.4. Cell Treatment. Raw 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% v/v fetal bovine serum (Gibco), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/mL streptomycin (Gibco). Cells were grown at 37°C in an atmosphere containing 5% CO_2 of 95% humidity. Cells were seeded in 6-well plates and treated with 1, 10, 100, 250, 500, and 1000 μg/mL of SSHT in the presence of LPS 1 μg/mL for 24 h. DEX was treated at the 1 μg/mL concentration to raw 264.7 cells for 24 h.

2.5. Preparations of Protein Extracts. Whole left lobe of lung tissues containing the left-upper lobe and the lower lobe was homogenized using a radioimmunoprecipitation assay (RIPA) lysis buffer (50 mm Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 150 mM NaCl) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) for extracts of whole proteins. The lysed proteins were centrifuged at 15,928 g for 10 min at 4°C and collected supernatant was used to determine mitogen-activated protein (MAP) Kinase, ERK1/2, SAPK/JNK, and p38. To extract cytoplasmic proteins, frozen lung tissues were homogenized with cytoplasmic buffer (10 mM HEPES, pH 7.9, 20 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.15% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, and 5 mM Na3VO4) containing the protease inhibitor cocktail and centrifuged at 24 g for 5 min. The supernatants obtained from the homogenate are used to analyze IκB-α and phospho-IκB-α. For nuclear protein extraction, a nuclear lysis buffer (20 mM HEPES, pH 7.9,

400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5% Nonidet P-40, 50 mM ß-glycerophosphate, 10 mM NaF, and 5 mM Na3VO4) containing a protease inhibitor cocktail was added to the pellet. Nuclear protein was centrifuged at 15,928 g for 10 min to detect NF-κB.

2.6. Western Blotting Analysis. The amount of proteins was quantified using the Bradford protein assay. Protein samples (20 μg/lane) were separated into 10% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked for 1 h in TBS containing 0.1% Tween (TBS-T) and 5% bovine serum albumin. The membrane was incubated overnight at 4°C with primary antibodies. The following antibodies were used in this study: ß-actin (1:1000; cat. no. sc-47778; Santa Cruz Biotechnology), ERK1/2 (1:1000; cat. no. 4695S; Cell Signaling Technology), phospho-ERK1/2 (1:1000; cat. no. 4370S; Cell Signaling Technology), SAPK/JNK (1:1000; cat. no. 9252S; Cell Signaling Technology), phospho-SAPK/JNK (1:1000; cat. no. sc-293138; Santa Cruz Biotechnology), p38 (1:1000; cat. no. 9212; Cell Signaling Technology), phospho-p38 (1:1000; cat. no. sc-166182; Santa Cruz Biotechnology), NF-κB (1:1000; cat. no. 3034S; Cell Signaling Technology), IκB-α (1:1000; cat. no. sc-1643; Santa Cruz Biotechnology), and phospho-IκB-α (1:1000; cat. no. sc-8404; Santa Cruz Biotechnology). Anti-mouse IgG (1:3000; cat. no. sc-516102; Santa Cruz Biotechnology) and anti-rabbit IgG (1:4000; cat. no. sc-2357; Santa Cruz Biotechnology) secondary antibodies were incubated room temperature for 1 h. The blots were visualized by Davinch-Chemi (cat no. CAS400 MF; Davinch-K, Seoul, Korea) with enhanced chemiluminescence kit (cat. no. ABC-3001; AbClon, Seoul, Korea).

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis. For RNA extraction, the lung tissues and raw 264.7 cells were homogenized in TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. The optical density was read at a wavelength of 260 nm to quantify RNA. To synthesize complementary DNA, Maxime RT PreMix (Invitrogen) was mixed with 1 μg RNA at 45°C for 1 hour and incubated at 95°C for 5 min. PCR was performed using Maxime PCR PreMix (Invitrogen) and synthesized cDNA template. The sequence of the primers used was as shown in Table 1. The amplification conditions were 94°C for 30 sec, followed by 40 cycles of 94°C for 1 min, range from 58 to 70°C for 30 sec, 72°C for 1 min and final extension at 72°C for 5 min. The relative expressions of gene were calculated and normalized to GAPDH and analyzed by ImageJ software.

2.8. Statistical Analysis. Significance was determined by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Effects of SSHT on the Histological Analysis of Lung Tissue. Histological analysis was conducted to evaluate whether SSHT recovers LPS-induced change of lung structure. Compared to the structure of lung tissues in normal group, the LPS group led to alveolar hemorrhage, alveolar wall thickening, and alveolar spaces shrinkage. In contrast, SSHT treatment relieved alveolar wall thickness and alveolar spaces shrinkage similar to normal group (Figure 1(a)). The ALI score by LPS sensitization was 3.5 times higher than nontreated CTR group ($p < 0.05$). SSHT treatment at all concentrations (0.05, 0.55, and 5.55 g/kg) significantly decreased the histology scores by 34.5%, 46.2%, and 42.2% ($p < 0.05$), respectively, while DEX treatment showed 51.8% reduction ($p < 0.05$) of ALI score in LPS-sensitized mice (Figure 1(b)).

3.2. Effects of SSHT on Expression of Inflammatory Cytokines IL-6, TNF-α, and IFN-γ in Lung Tissues and Raw 264.7 Cells. To examine whether SSHT treatment inhibits LPS-induced inflammation in ALI model, proinflammatory cytokines IL-6, TNF-α, and interferon-γ (IFN-γ) in lung tissue was assessed by RT-PCR. Expression of IL-6 was increased 3.18 times ($p < 0.05$) in LPS-treated group compared to the normal group. SSHT treatment at the concentrations of 0.55 and 5.55 g/kg decreased expression of IL-6 to 34.1% and 55.4% of LPS-treated group, respectively ($p < 0.05$). The levels of TNF-α increased 3.56 times of LPS-treated group than that in the normal group ($p < 0.05$). Expression of TNF-α was decreased 58.5% in treated with 5.55 g/kg of SSHT ($p < 0.05$). In contrast with the other cytokine expression levels, expression of IFN-γ was 0.57 times decreased in LPS-treated group compared with the nontreated CTR group ($p < 0.05$). SSHT treatment with 5.55 g/kg increased the expression of IFN-γ 35.94% compared to LPS group (Figure 2(a)) ($p < 0.05$).

Additionally, the expressions of IL-6 and TNF-α in LPS-sensitized raw 264.7 cells were 2.8 and 3.0 times increased compared to nontreated cells ($p < 0.05$), while LPS significantly decreased the mRNA level of IFN-γ in raw 264.7 cells ($p < 0.05$). In the SSHT-treated cells in the presence of LPS, the expressions of IL-6 and TNF-α were markedly decreased. 500 and 1000 μg/mL of SSHT significantly inhibited the IL-6 levels, while 250, 500, and 1000 μg/mL of SSHT significantly reduced the TNF-α levels, respectively ($p < 0.05$). Moreover, 1000 μg/mL of SSHT significantly increased the IFN-γ expression in LPS-sensitized raw 264.7 cells (Figure 2(b)) ($p < 0.05$).

3.3. Effects of SSHT on NF-κB Translocation and IκB-α Phosphorylation in Lung Tissue. IκB-α/NF-κB pathway is a key regulator of inflammation and highly activated in ALI, while LPS treatment induced the increases of 1.24 times on phosphorylated IκB-α and 1.66 times on translocated NF-κB, respectively ($p < 0.05$). IκB-α phosphorylation was reduced 18.6% and 24.3% by 0.55 and 5.55 g/kg of SSHT treatment, respectively (Figure 3) ($p < 0.05$). Moreover,

Table 1: Sequence of reverse transcription PCR primers.

Target gene	$5' \longrightarrow 3'$ forward primer	$5' \longrightarrow 3'$ reverse primer
IL-6	CGGAGAGGAGACTTCACAGAGGA	GGAGAGCATTGGAAATTGGGG
TNF-α	CCTGTAGCCCACGTCGTAGC	TTGACCTCAGCGCTGAGTTG
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG
GAPDH	GGCATGGACTGTGGTCATGA	TTCACCACCATGGAGAAGGC

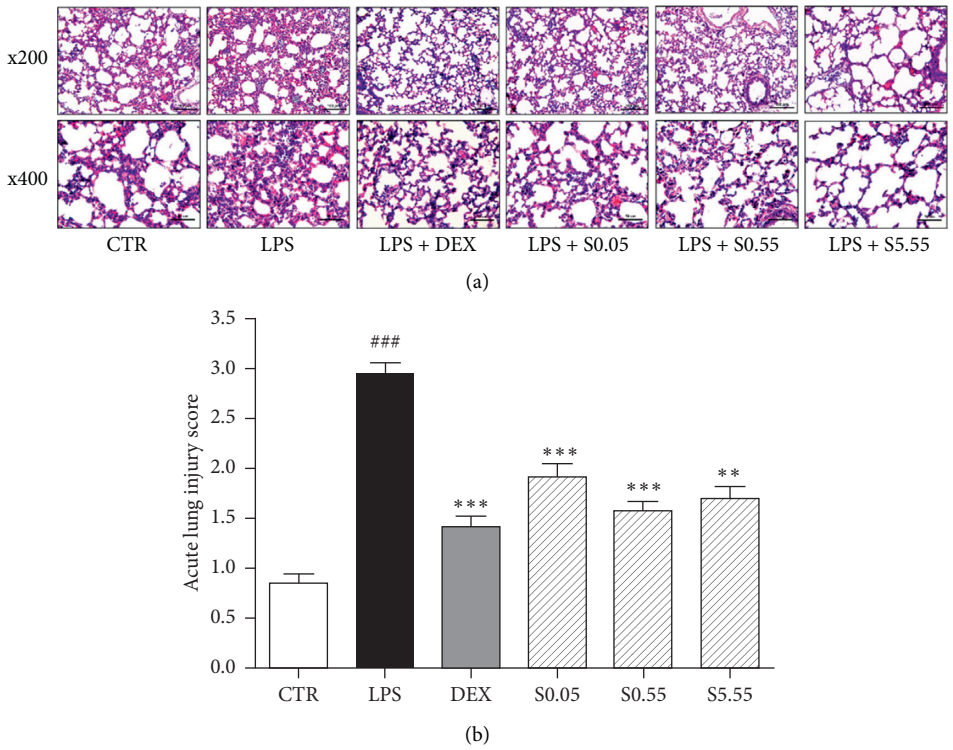

Figure 1: Histological changes of lung tissues indicated by hematoxylin and eosin staining. Representative images of lung tissues (a) and ALI score (b). The sections were stained with hematoxylin and eosin (H&E). Original magnifications were 200× and 400×. CTR: normal control group; LPS: LPS-sensitized ALI group; DEX: DEX-treated and LPS-sensitized group; S0.05: 0.05 g/kg SSHT-treated and LPS-sensitized group; S0.55: 0.55 g/kg SSHT-treated and LPS-sensitized group; and S5.55: 5.55 g/kg SSHT-treated and LPS-sensitized group. Results are presented as mean ± S.E.M. $^{###}p < 0.001$ compared with CTR group; $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with the LPS group.

expression of nuclear NF-κB was also decreased 63.5%, 67.9%, and 88.9% by SSHT treatment ($p < 0.05$).

3.4. Effects of SSHT on Expression of MAP Kinase in Lung Tissue. LPS-induced 1.36 times increase of ERK phosphorylation in lung tissues of mice ($p < 0.05$). Administration of SSHT at all concentrations (0.05, 0.55, and 5.55 g/kg) had significantly dose-dependently decreased the ERK phosphorylation by 13%, 26.4%, and 56.8% ($p < 0.05$). Phosphorylation of JNK was significantly increased by 2.65 times in LPS-treated group compared with the CTR group ($p < 0.05$), whereas 0.55 and 5.55 g/kg of SSHT treatment markedly reduced LPS-induced phosphorylated JNK by 33.7% and 41% ($p < 0.05$). In LPS-sensitized mice, phosphorylation of p38 was 1.12 times increased in comparison with normal mice ($p < 0.05$). SSHT 5.55 g/kg-treated group showed a significant 15.7% reduction of p38 phosphorylation ($p < 0.05$) (Figure 4).

4. Discussion

New finding of efficacy of medicine would be helpful to expand its medical indication as a prescription for health insurance. Recommended daily intake dose of SSHT to human is 9 g/day/60 kg, converted to 5.55 g/kg of SSHT in mice. In this study, 0.05, 0.55, and 5.55 g/kg of SSHT were administered to LPS-induced ALI mice. LPS causes histological changes including alveolar hemorrhage, shrinkage of alveolar spaces, and alveolar wall thickening [25]. Those symptoms can be prevented by treatment with SSHT at a concentration of 5.55 g/kg.

B. falcatum Linne polysaccharides, a major herb of SSHT, suppressed LPS-induced proinflammatory cytokines production including IL-6, IL-1β, IFN-β, and TNF-α in peritoneal macrophages of mice [26]. Additionally, baicalin derived from *S. baicalensis* Georgi consisting of SSHT has been studied to decrease TNF-α and IL-1β levels in

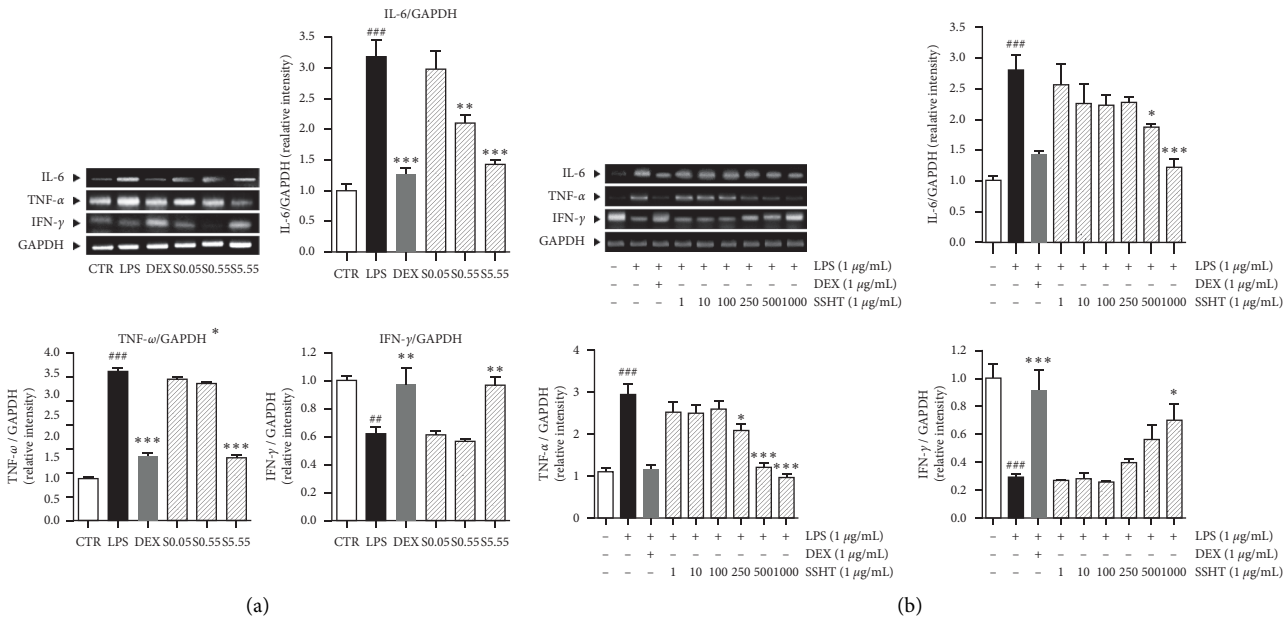

Figure 2: Expressions of cytokines in lung tissues (a) and raw 264.7 cells (b). CTR: normal control group; LPS: LPS-sensitized ALI group; DEX: DEX-treated and LPS-sensitized group; S0.05: 0.05 g/kg SSHT-treated and LPS-sensitized group; S0.55: 0.55 g/kg SSHT-treated and LPS-sensitized group; and S5.55: 5.55 g/kg SSHT-treated and LPS-sensitized group. Results are presented as mean ± S.E.M. $^{###}p < 0.001$ and $^{##}p < 0.01$ compared with CTR group and nontreated cells; $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with the LPS group and LPS-sensitized cells.

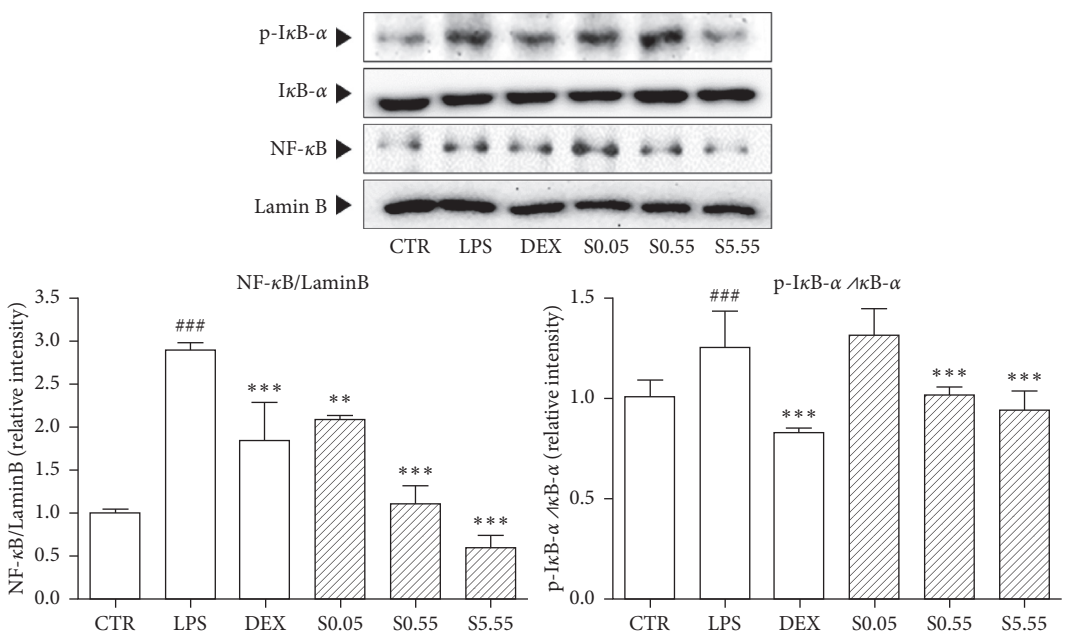

Figure 3: Expression of nuclear NF-κB and cytoplasmic IκB-α of lung tissues indicated by western blot analysis. CTR: normal control group; LPS: LPS-sensitized ALI group; DEX: DEX-treated and LPS-sensitized group; S0.05: 0.05 g/kg SSHT-treated and LPS-sensitized group; S0.55: 0.55 g/kg SSHT-treated and LPS-sensitized group; and S5.55: 5.55 g/kg SSHT-treated and LPS-sensitized group. Results are presented as mean ± S.E.M. $^{###}p < 0.001$ compared with the CTR group; $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with the LPS group.

bronchoalveolar lavage fluids of LPS-induced ALI mice [27]. Total saponin from Ginseng as another major herb of SSHT was reported to inhibit LPS-induced increase of serum TNF-α level [28]. Based on the previous evidence, we anticipated that SSHT has inhibitory effects against ALI by regulating proinflammatory cytokines. In particular, lung proinflammatory cytokine expressions such as IL-6, TNF-α, and IFN-γ in ALI model were investigated to find the underlying

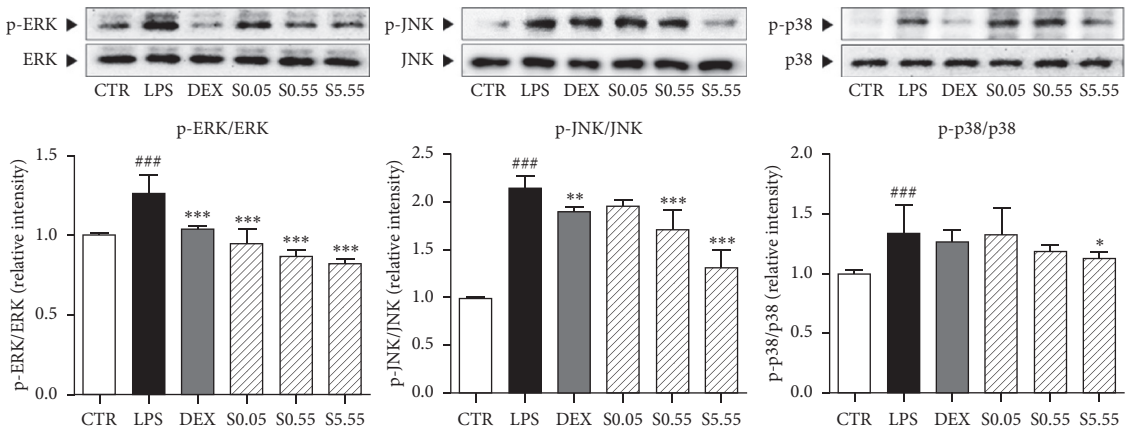

Figure 4: Expressions of MAP kinase of lung tissues indicated by western blot analysis. CTR: normal control group; LPS: LPS-sensitized ALI group; DEX: DEX-treated and LPS-sensitized group; S0.05: 0.05 g/kg SSHT-treated and LPS-sensitized group; S0.55: 0.55 g/kg SSHT-treated and LPS-sensitized group; and S5.55: 5.55 g/kg SSHT-treated and LPS-sensitized group. Results are presented as mean ± S.E.M. $^{###}p < 0.001$ compared with the CTR group; $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with the LPS group.

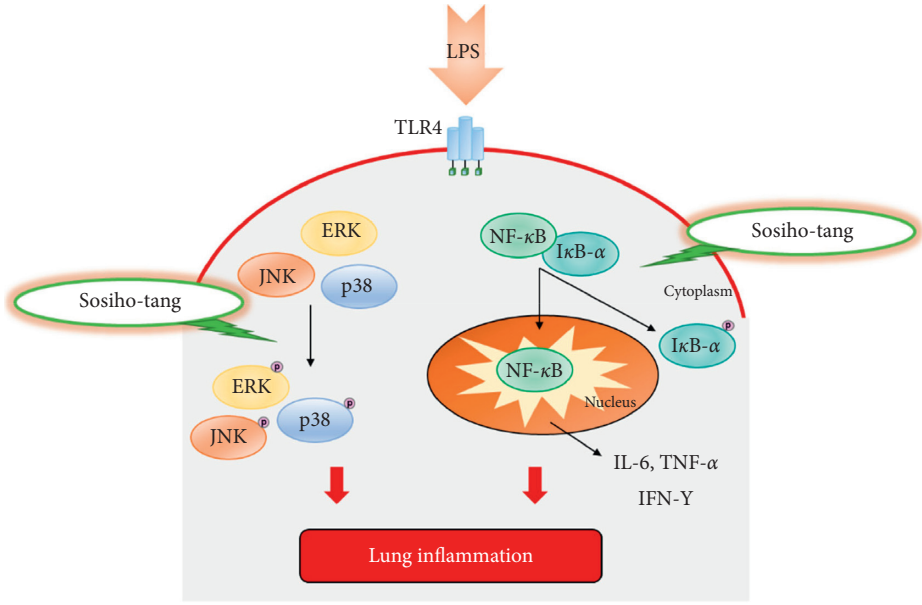

Figure 5: Schematic diagram of potential action of SSHT on lung inflammation. SSHT appears to regulate the IL-6, TNF-α, and IFN-γ, following the inhibition of NF-κB and MAP kinases expressions. These actions inhibit the LPS-induced lung inflammation in ALI.

mechanism of SSHT on the recovery of alveolar wall thickening. Proinflammatory cytokines such as IL-6 and TNF-α are considered key components of the acute inflammatory response [29–31]. Some studies have shown that TNF-α produced by initiating inflammatory response in macrophages undergo to activate NF-κB pathway and MAP kinase [32]. IL-6 is a multifunctional cytokine produced by monocytes and macrophages in the LPS-induced immune response [33]. IFN-γ as a primary activator by stimulating innate and adaptive immunity clears intracellular pathogens and produces enzymes to inhibit viral infections [34, 35]. IL-6 and TNF-α mRNA levels in the lung tissues and raw 264.7 cells were increased by exposure of LPS and significantly decreased by the pretreatment or cotreatment with SSHT. Those results demonstrate that SSHT might regulate the counterbalance of inflammatory responses by down-regulating IL-6 and TNF-α and upregulating IFN-γ.

NF-κB is translocated from the cytoplasm to the nucleus by inflammatory cytokines such as IL-6 and TNF-α [36–40]. The nuclear translocation of NF-κB is regulated by IκB-α proteolytic degradation [41]. Additionally, MAP kinases including ERK12, SAPK/JNK, and p38 are associated with the production of cytokines in the inflammatory response [42]. Inhibition of NF-κB and MAP kinases in LPS-sensitized lung tissues could ameliorate ALI [43]. The expression of NF-κB and the phosphorylation of IκB-α in LPS-sensitized lung tissue were increased, but they were markedly decreased in the SSHT-treated mice. SSHT also significantly reduced the phosphorylation of ERK1/2, SAPK/JNK, and p38 in the lungs. In addition to the results from IL-6 and

TNF-α, SSHT attenuated the activation of MAP kinase and NF-κB by inhibition of inflammatory cytokines such as IL-6 and TNF-α (Figure 5).

5. Conclusion

In conclusion, SSHT alleviated the inflammatory IL-6 and TNF-α production, following the decreases of transcription factors including NF-κB and MAP kinases. Inhibition of inflammatory responses by SSHT in lung would be helpful to ameliorate LPS-induced ALI. Taken together, SSHT might be beneficial for treating ALI. Since the administration of 5.55 g/kg of SSHT to mice is converted to daily intake 9 g/day/60 kg dose for human, Jungwoo Pharmacy *Sosiho*-tang Soft Ext. is applicable for treating lung inflammation. Optimal dosage range of SSHT might be 9 g/day/60 kg, a daily intake dose for human in present. Further studies regarding the maximal efficacy and toxicity of SSHT are needed.

Abbreviations

ALI: Acute lung injury
HED: Human equivalent dose
IFN-γ: Interferon-γ
IL: Interleukin
LPS: Lipopolysaccharide
MAP: Mitogen-activated protein
SSHT: *Sosiho*-tang
TNF-α: Tumor necrosis factor-α.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Research Article

Uncovering the Anti-Lung-Cancer Mechanisms of the Herbal Drug FDY2004 by Network Pharmacology

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With growing evidence on the therapeutic efficacy and safety of herbal drugs, there has been a substantial increase in their application in the lung cancer treatment. Meanwhile, their action mechanisms at the system level have not been comprehensively uncovered. To this end, we employed a network pharmacology methodology to elucidate the systematic action mechanisms of FDY2004, an anticancer herbal drug composed of Moutan Radicis Cortex, Persicae Semen, and Rhei Radix et Rhizoma, in lung cancer treatment. By evaluating the pharmacokinetic properties of the chemical compounds present in FDY2004 using herbal medicine-associated databases, we identified its 29 active chemical components interacting with 141 lung cancer-associated therapeutic targets in humans. The functional enrichment analysis of the lung cancer-related targets of FDY2004 revealed the enriched Gene Ontology terms, involving the regulation of cell proliferation and growth, cell survival and death, and oxidative stress responses. Moreover, we identified key FDY2004-targeted oncogenic and tumor-suppressive pathways associated with lung cancer, including the phosphatidylinositol 3-kinase-Akt, mitogen-activated protein kinase, tumor necrosis factor, Ras, focal adhesion, and hypoxia-inducible factor-1 signaling pathways. Overall, our study provides novel evidence and basis for research on the comprehensive anticancer mechanisms of herbal medicines in lung cancer treatment.

1. Introduction

Despite the advances in anticancer therapies, lung cancer (LC) remains the most common reason for cancer mortality, which accounts for 18.4% of global cancer deaths [1]. Accumulating evidence and increasing understanding of LC pathology have led to the development of effective anticancer therapies such as chemotherapy, targeted therapy, and cancer immunotherapy that can prolong the survival of patients with LC; however, these therapies may frequently and inevitably accompany therapeutic resistance and toxic adverse effects [2, 3]. Therefore, there has been a substantial increase in the application of herbal drugs in cancer treatment owing to their potent anticancer effects and less toxicities [4–6]. It has been shown that the administration of herbal drugs can enhance the efficacy and attenuate the adverse effects of anticancer therapies, alleviate cancer symptoms, and improve the survival and clinical outcomes of patients with cancer [6–8].

FDY2004 is a herbal drug composed of three herbal medicines, namely, Moutan Radicis Cortex (MRC), Persicae Semen (PS), and Rhei Radix et Rhizoma (RRR) (Supplementary Table S1) [9]. This herbal drug may exert potent antiproliferative effect against LC cells (Supplementary Table S1) [9]; however, its system-level anticancer mechanisms in LC treatment remain to be elucidated.

With advances in scientific and analytical technologies, various convergence research methodologies such as network pharmacology have emerged [5, 10–12]. They have been used to investigate complex multiple compound-multiple target pharmacological mechanisms of herbal drugs [5, 10–12]. Network pharmacology is used to explore the

mechanisms underlying various diseases and action mechanisms of therapeutic herbal drugs [5, 10–12]. It involves the interactions among biological elements, such as genes, proteins, and metabolites, and integrates pharmacology, medicine, and network biology [5, 10–12]. This research strategy has been proven beneficial in understanding the multiple compound-multiple target mechanisms of herbal drugs via the following: (i) investigation of their active chemical components and disease-associated therapeutic targets and (ii) analysis of their therapeutic functions to uncover the polypharmacological mechanisms of herbal medicines [5, 10–12]. By employing a network pharmacology methodology, we uncovered the anti-LC mechanisms of FDY2004.

2. Materials and Methods

2.1. Investigation of the Active Chemical Components of FDY2004. We retrieved the chemical components of FDY2004 from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) and Anticancer Herbs Database of Systems Pharmacology (CancerHSP) databases [13, 14]. We then used the pharmacokinetic characteristics obtained from the aforementioned databases [13, 14], including oral bioavailability, Caco-2 permeability, and drug-likeness, to determine the active chemical components of FDY2004. Oral bioavailability is a measure of an orally administered drug's capacity to be transported to the general circulation and sites of drug action [13, 15]. Chemical compounds whose oral bioavailability is higher than 30% are regarded to possess effective absorption abilities [13, 15]. Caco-2 permeability is an indicator of the diffusion ability of a chemical compound in the intestinal epithelium, assessed using Caco-2 human intestinal cells [13]. Compounds with a Caco-2 permeability of ≥ -0.4 are considered to have effective permeability in the intestines [16, 17]. Drug-likeness is an index used to investigate the druggability of a chemical component with respect to its biochemical and physical properties using Tanimoto coefficients [13, 18]. The average drug-likeness of available drugs is 0.18; therefore, chemical compounds whose drug-likeness is higher than this average value are regarded to have druggable potential in network pharmacology analysis [13, 18]. Consequently, in this study, chemical components that meet the following criteria were determined to be bioactive: oral bioavailability $\geq 30\%$, drug-likeness ≥ 0.18, and Caco-2 permeability ≥ -0.4 [11, 13].

2.2. Identification of the Targets of Active Chemical Components. We retrieved the simplified molecular-input line-entry system (SMILES) notation for individual chemical components from the PubChem database [19]. By importing the SMILES information into diverse *in silico* tools, involving the SwissTargetPrediction [20], Search Tool for Interactions of Chemicals 5 [21], PharmMapper [22], and Similarity Ensemble Approach [23], the human targets of FDY2004 were obtained. The LC-associated human targets were searched using "Lung Neoplasms" (Medical Subject Headings Unique ID: D008175) as a keyword in the following comprehensive genomic databases: Comparative Toxicogenomics Database [24], Therapeutic Target Database [25], Human Genome Epidemiology Navigator [26], GeneCards [27], DisGeNET [28], DrugBank [29], Online Mendelian Inheritance in Man [30], and Pharmacogenomics Knowledge Base [31].

2.3. Construction of Herbal Drug-Associated Networks. Herbal medicine-active chemical component (H-C), active chemical component-target (C-T), and target-pathway (T-P) interaction networks were built by connecting the herbal components of FDY2004 with their active chemical components, the components with their interacting targets, and the targets with their relevant enriched pathways, respectively. A protein-protein interaction (PPI) network was built based on the interaction data for the targets obtained from the STRING database (interaction confidence score ≥ 0.7) [32]. Network visualization and analysis were conducted using Cytoscape [33]. In the network pharmacology analysis, individual constituents relevant to a herbal drug, including its herbal medicines, chemical components, targets, and pathways, are represented as nodes [34]. Their interactions are represented as links (or edges) [34]. The degree of nodes is defined as the number of their links, and nodes with a relatively high degree are called hubs [34].

2.4. Survival Analysis. The correlation between the expression status of FDY2004 targets and the survival of patients with LC was analyzed using Kaplan–Meier plotter [35].

2.5. Functional Enrichment Analysis. Functional enrichment of Gene Ontology (GO) terms and pathways for "*Homo sapiens*" by FDY2004 targets was analyzed using g:Profiler [36] and Kyoto Encyclopedia of Genes and Genomes [37].

2.6. Molecular Docking Analysis. We obtained the structures of the chemical components and their targets from PubChem [19] and RCSB Protein Data Bank [38], respectively. Then, we imported the collected structural information into Autodock Vina [39] and analyzed the docking scores of individual chemical component-target pairs. As reported previously, we considered that a chemical component-target pair might have a high binding affinity if it has a docking score of less than -5.0 [40, 41].

3. Results

3.1. Active Chemical Components of FDY2004. From the TCMSP and CancerHSP [13, 14], we obtained detailed information on the chemical components of FDY2004 (Supplementary Table S2). The chemical components that satisfied the following criteria were considered bioactive: oral bioavailability $\geq 30\%$, drug-likeness ≥ 0.18, and Caco-2 permeability ≥ -0.4 [11, 13]. We also considered numerous compounds as active compounds because of their high amounts and potent activity, although they did not meet the aforementioned criteria [42–56]. Thus, 35 bioactive chemical

compounds of FDY2004 were identified (Supplementary Table S3).

3.2. Targets of the Active Chemical Components of FDY2004. We obtained 212 human molecular targets for the 29 bioactive chemical components of FDY2004 (see Materials and Methods) (Supplementary Table S4). The information on the LC-associated human genes and proteins was retrieved from various genomic databases (see Materials and Methods), and 141 of all the 212 FDY2004 targets were considered LC-related targets.

3.3. Network Pharmacological Identification of the Action Mechanisms of FDY2004. By integrating the comprehensive data of FDY2004, including its herbal and chemical components and their LC-related targets, we built a herbal medicine-active chemical component-target (H-C-T) network representing the polypharmacological features of the herbal drug (Figure 1). The network consisted of 173 nodes (3 herbal medicines, 29 bioactive chemical components, and 141 targets) and 304 links (Figure 1 and Supplementary Table S4). In this network, quercetin, kaempferol, gallic acid, emodin, campesterol, (+)-epicatechin, (+)-catechin, and (−)-catechin had the highest number of interacting targets (Figure 1), demonstrating that they may be the key pharmacological compounds underlying the anti-LC effects of FDY2004. It is noteworthy that 96 of the 141 LC-related genes/proteins were common therapeutic targets of two or more bioactive chemical components of FDY2004 (Figure 1), implying the polypharmacological and coordinated action mechanisms of FDY2004.

To understand the interactions among the LC-related targets of FDY2004, a PPI network with 114 nodes and 304 edges was generated, where the targets served as nodes and their interactions represented edges (Figure 2). We then searched for nodes with a relatively high degree (i.e., hubs) [57, 58]. They are reported to have key roles in the pharmacological activities of drugs and serve as potential therapeutic targets [57, 58]. As reported previously, hubs were defined as nodes with a degree higher than or equal to twice the average degree of all nodes in a PPI network [11]. The nodes TP53, PIK3R1, HSP90AA1, AKT1, VEGFA, EGFR, JUN, PTK2, TNF, ESR1, NFKB1, and RAC1 were identified as hubs with high degree (Figure 2), demonstrating that these targets may be important for the exertion of anti-LC pharmacological effects of FDY2004. The expression status of these hub targets was further shown to be significantly related to the survival of patients with LC (Figure 3), implying their potential clinical significance and prognostic role.

3.4. Functional Enrichment Analysis of FDY2004-Associated Targets and Pathways. To explore the molecular mechanisms of FDY004 in LC treatment based on the biological functions of its targets, we carried out the GO enrichment analysis. The GO terms involved in the various biological functions, including cell proliferation and growth, cell survival and death, and oxidative stress responses, were enriched by the LC-related targets of FDY2004 (Supplementary Figure S1), indicating the anticancer molecular characteristics of its pharmacological activity.

To investigate the pathway-level pharmacological properties of FDY2004 against LC, we conducted the pathway enrichment analysis (Figure 4 and Supplementary Figure S1). The following signaling pathways were found to be enriched by the LC-associated targets of FDY2004: "Pathways in cancer," "PI3K-Akt signaling pathway," "MAPK signaling pathway," "TNF signaling pathway," "Ras signaling pathway," "Apoptosis," "Focal adhesion," "HIF-1 signaling pathway," "Cellular senescence," "EGFR tyrosine kinase inhibitor resistance," "Estrogen signaling pathway," "PD-L1 expression and PD-1 checkpoint pathway in cancer," "Small cell lung cancer," "Non-small cell lung cancer," "Platinum drug resistance," "ErbB signaling pathway," "p53 signaling pathway," and "VEGF signaling pathway" (Figure 4 and Supplementary Figure S1).

Together, the results suggest the system-level mechanisms of FDY2004 against LC from the molecular- and pathway perspectives.

3.5. Molecular Docking of the FDY2004 Targets. To investigate the binding activities of compound-target interactions for FDY2004, we analyzed their molecular docking affinities (see Materials and Methods). In the docking analysis, 95.19% of the active compound-target pairs presented docking scores of ≤ -5.0, implying the potential pharmacological binding activities of the herbal drug (Figure 5 and Supplementary Table S5).

4. Discussion

Although there has been increasing use of herbal drugs in LC treatment, their system-level anticancer mechanisms have not been comprehensively understood. Here, we employed a network pharmacological approach to uncover the therapeutic mechanisms of FDY2004 [9] in LC treatment from a system-level view. The network pharmacological investigation of FDY2004 revealed 29 active chemical components that interact with 141 lung cancer-associated therapeutic targets, mediating the anti-LC effects of the herbal drug. The GO enrichment analysis of the FDY2004 targets revealed the molecular action mechanisms of FDY2004, involving the regulation of cell proliferation and growth, cell survival and death, and oxidative stress responses. Furthermore, the key FDY2004-targeted oncogenic and tumor-suppressive pathways implicated in LC development and progression were the phosphatidylinositol 3-kinase (PI3K)-Akt, mitogen-activated protein kinase (MAPK), tumor necrosis factor (TNF), Ras, focal adhesion, and hypoxia-inducible factor (HIF)-1 signaling pathways.

The LC-related hub targets of FDY2004 were found to be closely associated with LC pathology and play a role as prognostic indicators for the survival and therapeutic sensitivity of patients with LC. The tumor suppressor *TP53* is one of the most frequently mutated and malfunctioned

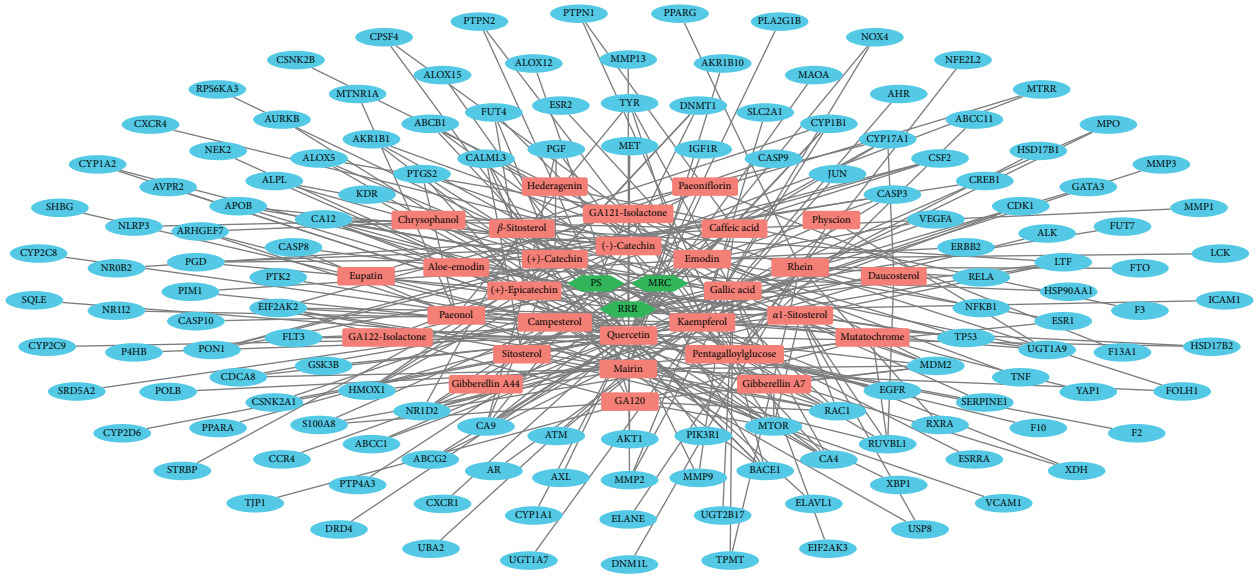

Figure 1: The herbal medicine-active chemical component-target network of FDY2004. Green hexagon nodes, herbal medicines; red rectangle nodes, active chemical compounds; blue eclipse nodes, lung cancer-related targets.

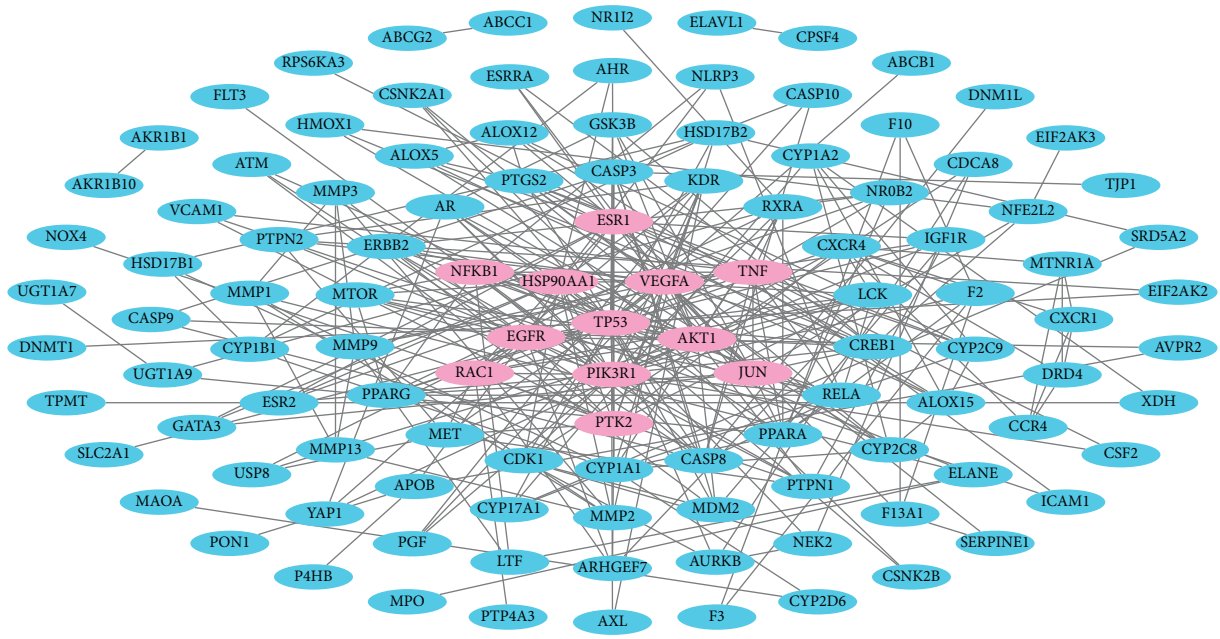

Figure 2: The protein-protein interaction network for lung cancer-related targets of FDY2004. Pink nodes, hub targets.

genes in the pathological process of LC, and its genetic and functional status may serve as a predictor for the risk, survival, and therapeutic outcomes of LC [59–63]. *PIK3R1* is involved in the regulation of LC cell growth [64]. The upregulation of *HSP90AA1* correlates with the occurrence, progression, and clinical outcomes of LC, and its inhibition can repress the proliferation, survival, and metastasis of LC cells [65, 66]. The abnormal regulation of Akt1 (encoded by *AKT1*) and TNF-α (encoded by *TNF*) may enhance the growth, survival, proliferation, metastasis, epithelial-to-mesenchymal transition (EMT), and stemness capacity of LC cells, and they are potential targets that can alleviate chemotherapy and radiotherapy resistance [67–76]. Clinical studies have also reported that *AKT1* and *TNF* may be prognostic determinants for patients' survival and treatment outcomes with LC [73, 77–80]. Vascular endothelial growth factor (VEGF)-A (encoded by *VEGFA*) enhances the metastasis and angiogenesis of LC cells and thereby contribute to the progression of LC, and its activation profile is related to a poor clinical prognosis and the survival of patients with LC [81–86]. Dysregulated expression of *EGFR* and its encoded receptor tyrosine kinase activity may lead to the induction of various cancerous cellular processes underlying the LC pathology,

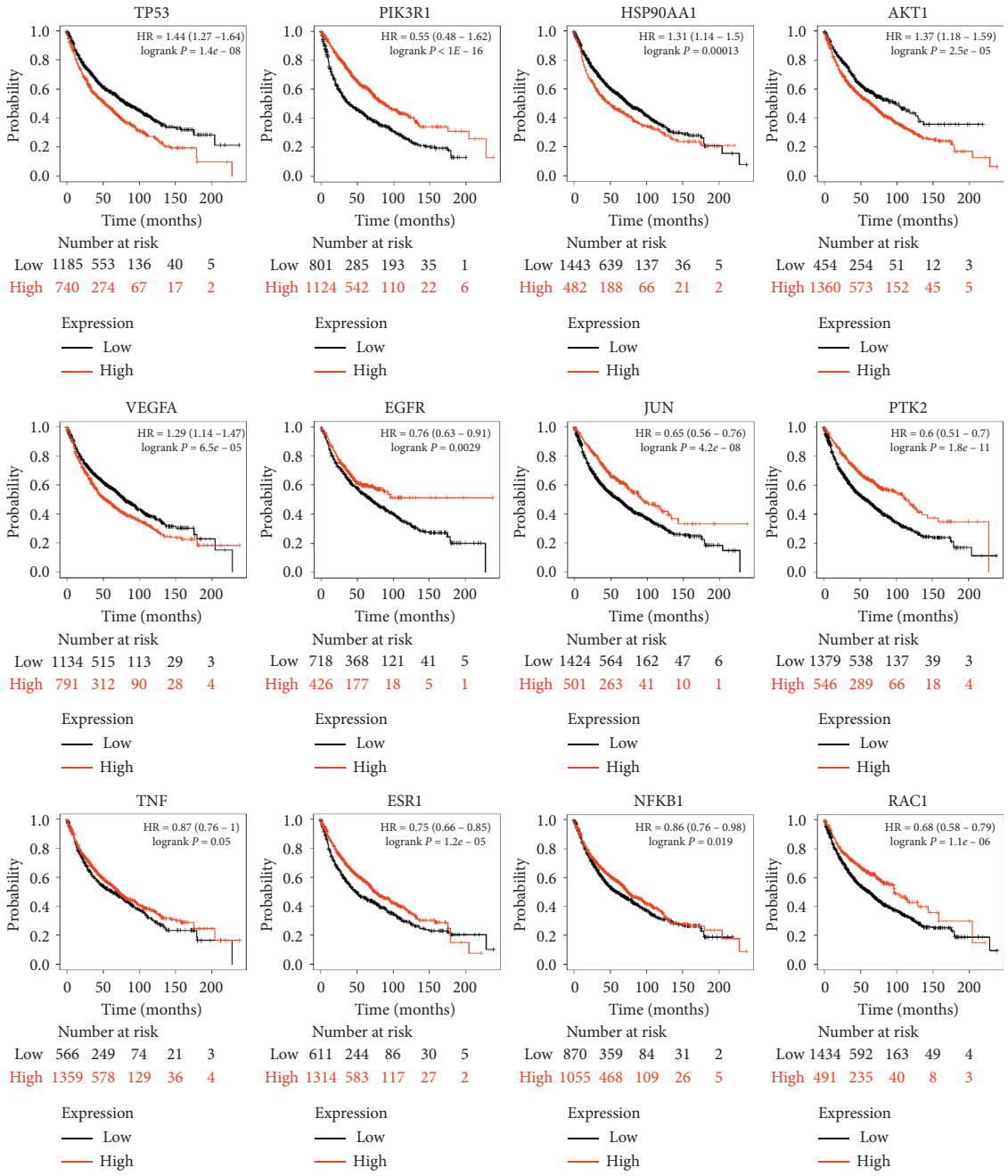

Figure 3: Survival analysis of lung cancer-related hub targets of FDY2004. Kaplan–Meier curves for overall survival of the patients with lung cancer with respect to the expression of the indicated targets.

making it a key target of widely used antitumor agents against LC in clinical settings [87–89]. c-Jun (encoded by *JUN*) functions as a modulator of the growth, proliferation, and apoptosis of LC cells as well as a mediator of the pharmacological effects of cytotoxic drugs [90–92]. Pharmacological modulation of focal adhesion kinase (FAK; encoded by *PTK2*) and Ras-related C3 botulinum toxin substrate 1 (RAC1; encoded by *RAC1*) reduces the proliferation, migration, invasion, EMT, motility, angiogenesis, and stemness activity of LC cells, and this reverses chemotherapy and radiotherapy resistance [93–103]. The expression of estrogen receptor (ER)-α (encoded by *ESR1*) might be correlated with the survival and prognosis of patients with LC, and previous studies have reported its role as a therapeutic target in LC treatment [104, 105]. The polymorphisms of *NFKB1* are associated with the risk of LC occurrence [106].

The signaling pathways targeted by FDY2004 are known to function as crucial regulators of LC development and progression, mediate treatment resistance to anticancer therapies, and play a role as therapeutic targets. The PI3K-Akt, MAPK, Ras, focal adhesion, HIF-1, and erythroblastic leukemia viral oncogene homolog (ErbB) signaling pathways coordinate diverse tumorigenic processes of cancer

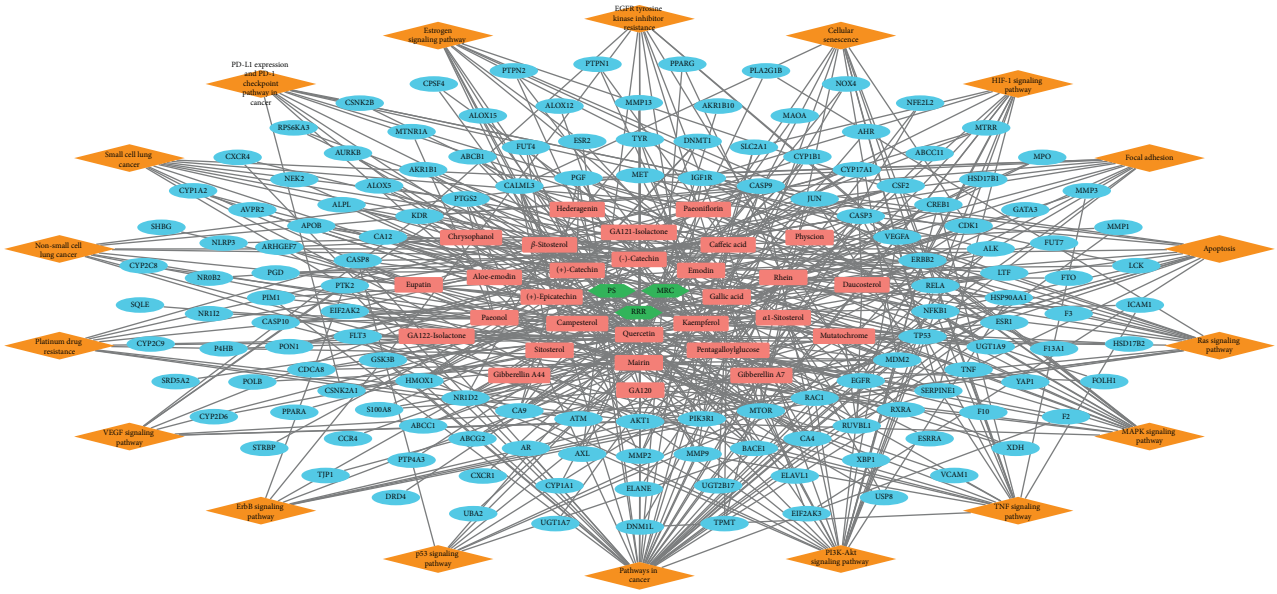

Figure 4: The herb-compound-target-pathway network of FDY2004. Green hexagon nodes, herbal medicines; red rectangle nodes, active chemical compounds; blue eclipse nodes, lung cancer-related targets; orange diamond nodes, signaling pathways.

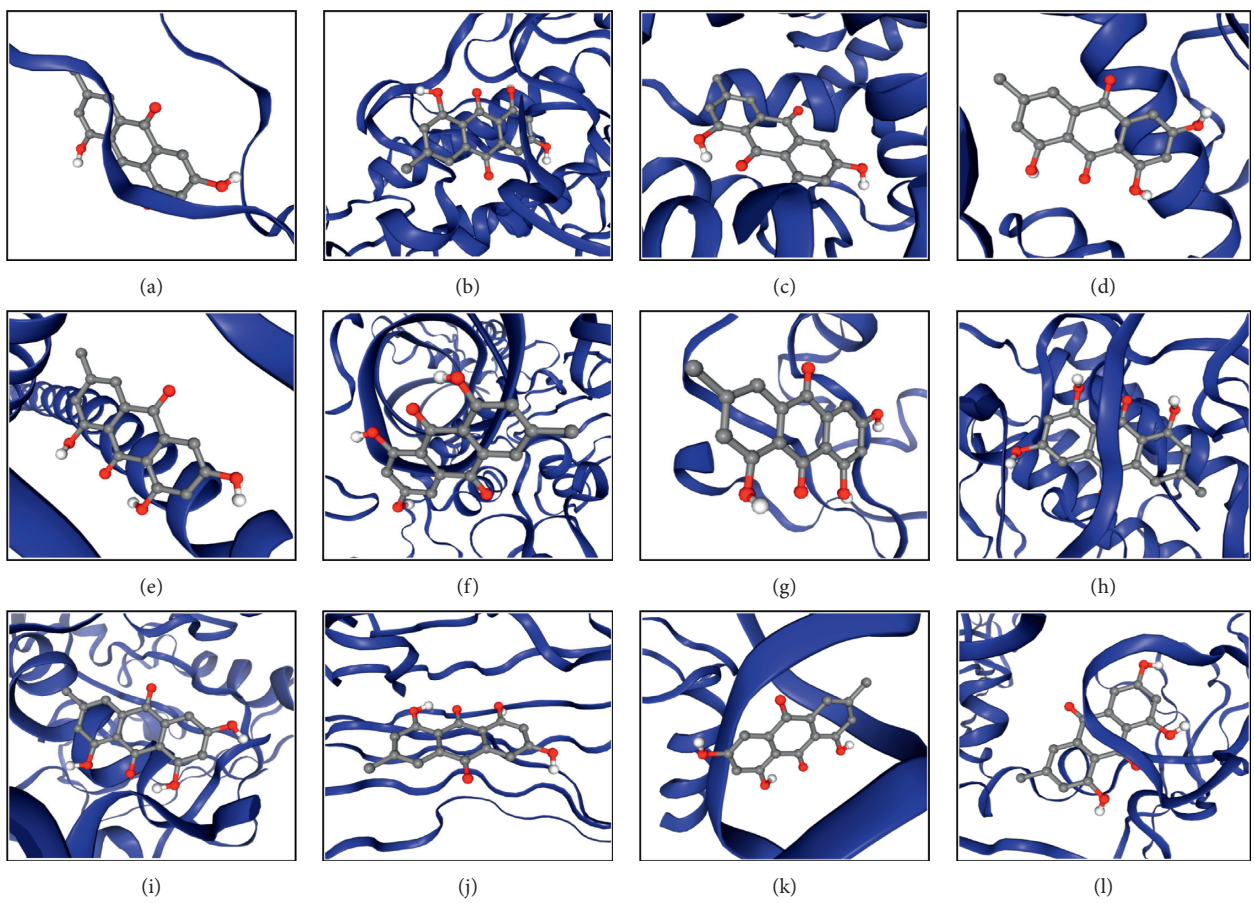

Figure 5: Molecular docking analysis for the active chemical compounds of FDY2004 and the targets. The analysis results of quercetin and the hub targets are shown as representatives. (a) Quercetin-AKT1 (score = −6.4). (b) Quercetin-EGFR (score = −7.9). (c) Quercetin-ESR1 (score = −6.5). (d) Quercetin-HSP90AA1 (score = −6.1). (e) Quercetin-JUN (score = −5.4). (f) Quercetin-NFKB1 (score = −6.6). (g) Quercetin-PI3KR1 (score = −6.3). (h) Quercetin-PTK2 (score = −6.1). (i) Quercetin-RAC1 (score = −9.3). (j) Quercetin-TNF (score = −6.5). (k) Quercetin-TP53 (score = −7.0). (l) Quercetin-VEGFA (score = −7.2).

cells, involving cell proliferation and growth, survival and cell death, anoikis resistance, metastasis, EMT, self-renewal potential and stemness properties, and angiogenesis, of LC cells [101, 102, 107–125]. In addition, aberrant regulations of these signaling pathways may contribute to therapeutic resistance, which can be overcome by genetic and pharmacological interventions of their activities [101, 102, 107–125]. The TNF signaling pathway is a key inflammation mediator involved in the development, progression, metastasis, and recurrence of LC, and the pathway constituents have prognostic significance in the clinical outcome of patients with LC [74–76, 126, 127]. The estrogen pathway and its components may possess carcinogenic properties in LC and act as potential targets [128–131]. The programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway is involved in the regulation of tumor-related immune processes, and it is a key target of cancer immunotherapy, which attempts to suppress immune escape and enhance antitumor immunity for the durable regression of malignant tumors of LC [132–134]. The dysfunction of genes and proteins comprising the p53 pathway, one of the common carcinogenic causes, is associated with various cancerous behaviors of LC cells, such as uncontrolled proliferation, survival, and cell cycle progression [61, 63, 135–143]. The genetic and functional activities of the pathway components might be correlated with the survival and anticancer therapeutic sensitivity of patients with LC [61, 63, 135–137, 139, 141–143]. The VEGF pathway may induce the progression of LC tumors by activating malignant angiogenic, metastatistic, and proliferative programs of cancer cells, and it is the primary pharmacological target of antiangiogenic anticancer drugs [144–146]. Defects in the regulation of important cellular phenotypes such as apoptosis and cellular senescence are the major drivers of the development and progression of LC, and their proper regulation is the key mechanism of anticancer therapeutics [147–152]. Resistance to platinum-based chemotherapeutics and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors is mediated by diverse oncogenic signaling mechanisms, and co-targeting the resistance-associated pathways may enhance the efficacy of LC treatment [153–157].

The active chemical components of FDY2004 have been reported to act as anticancer compounds in LC. Aloe-emodin induces DNA damage, autophagy, and death of LC cells by regulating reactive oxygen species (ROS) generation and signaling activities of the PI3K/Akt/mammalian target of rapamycin, MAPK, protein kinase C (PKC), and caspase pathways [158–162]. It also functions as a photosensitizer that enhances irradiation-induced anoikis in LC cells [158–162]. Caffeic acid has been shown to improve the cytotoxicity of chemotherapeutics in LC cells [163]. Catechins may suppress the growth and promote cell cycle arrest of LC cells by inactivating proliferation-inducing oncogenic kinases and cell cycle regulators [164, 165]. Chrysophanol regulates the activation of oxidative stress responses and relevant signaling pathways to reduce the proliferation, migration, invasion, and survival potential of LC cells [166, 167]. Daucosterol disturbs redox homeostasis and cell cycle processes to elicit growth arrest and death of LC cells [168, 169]. Emodin inhibits cell proliferation and migration and promotes EMT, autophagic cell death, and cell cycle arrest coordinated by chemokine, endoplasmic reticulum (ER) stress, ROS, p53, cell cycle, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tribbles pseudokinase 3, and PKC signaling; it enhances the efficacy of anticancer drugs [159, 170–180]. The proapoptotic and chemosensitizing effects of gallic acid are mediated by the EGFR, PD-L1, ROS, NF-κB, caspase, janus kinase (JAK)-signal transducer and activator (STAT), and mitochondrial pathways [181–188]. Hederagenin exerts cytotoxic effects and further synergizes with chemotherapeutic agents in LC cells [189]. Kaempferol may block the growth, survival, EMT, and migration of LC cells and enhance anti-LC therapies [190–192]. Previous studies have reported the anticancer roles of mairin (betulinic acid) in inducing apoptosis, suppressing proliferation, and reversing drug resistance of LC cells [193–195]. The antiproliferative, antimetastatic, and cell cycle arrest activities of paeoniflorin are mediated by the modulation of the FAS pathways and macrophage activation [196, 197]. Paeonol represses the proliferation and bone metastasis of LC cells and also serves as a radiosensitizer by inhibiting the PI3K/Akt pathway to enhance their apoptosis [198, 199]. Physcion increases the pharmacological sensitivity of LC cells to cytotoxic drugs [200]. Rhein induces apoptosis while suppressing the proliferation of LC cells mediated by the modulation of the calcium, ER stress, and STAT3 pathways [201, 202]. Previous studies have reported the inhibitory roles of quercetin on the growth, survival, metastasis, and chemotherapy and radiotherapy resistance of LC cells via cancer pathways such as Akt, MAPKs, NF-κB, inflammation, and apoptotic caspase signaling [203–209]. β-Sitosterol inhibits cancerous autophagic, proliferative, survival, and cell cycle regulatory processes in LC cells [169, 210, 211]. These observations support the pharmacological mechanisms underlying the anti-LC effects of FDY2004.

Overall, our study presents novel and comprehensive insights into and evidence of the anti-LC effects of FDY2004. Further preclinical and clinical studies are warranted to investigate the action mechanisms of FDY2004 and evaluate the pharmacological effects of its combinatorial use with standard anticancer strategies such as chemotherapy, targeted therapy, cancer immunotherapy, and radiotherapy.

Data Availability

The data used to support the findings of this study are included within the article and Supplementary Materials file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplementary Figure S1: functional enrichment analysis for the lung cancer-related targets of FDY2004. Supplementary

Table S1: general information and reports on evidence of biological activities of FDY2004 and its herbal constituents. Supplementary Table S2: chemical components of FDY2004. Supplementary Table S3: active chemical components of FDY2004. Supplementary Table S4: Targets of active chemical components of FDY2004. Supplementary Table S5: docking scores between active chemical components of FDY2004 and the lung cancer-associated targets. (*Supplementary Materials*)

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