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ORIGINAL RESEARCH

In vitro Study of Fungus-Like Organisms *Peronospora ficariae* and *Wilsoniana bliti* as Potential Inhalant Allergens

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Purpose: Allergic conditions have surged to epidemic proportions globally, affecting almost 30% of people worldwide. Fungi are a significant source of allergens, contributing to up to 6% of respiratory illnesses in the general population. Nonetheless, the exact cause of respiratory allergies often remains elusive. This research sought to explore the potential of two fungus-like species from the Peronosporales (*Peronospora ficariae*) and Albuginales (*Wilsoniana bliti*) to induce a proinflammatory response in vitro models of the upper and lower respiratory tract.

Materials and Methods: BEAS-2B and A549 cell lines were used to mimic upper and lower respiratory epithelial cells. The cytotoxicity of fungal extracts was assessed using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and flow cytometry assays. Reactive oxygen species (ROS) production in the cells was measured through flow cytometry, while ELISA (enzyme-linked immunosorbent assay) tests were used to quantify the production of proinflammatory cytokines and metalloproteinases. Immunofluorescence techniques were utilized to evaluate markers of cell integrity.

Results: Although *W. bliti* extracts did not trigger notable inflammatory responses, *P. ficariae* significantly enhanced the production of proinflammatory cytokines IL-1β and GM-CSF in both cell lines, which are linked to allergic reactions. The rise in these cytokines and increased ROS production were associated with disrupting tight junction proteins, such as E-cadherin and occludin, in epithelial cells.

Conclusion: The results indicate that *P. ficariae* extracts have the potential to collectively compromise the epithelial barrier in the upper and lower respiratory tracts by inducing proinflammatory cytokines and promoting the production of reactive oxygen species and metalloproteinases. While none of these parameters were exceptionally high, their combined effect was observed to disrupt epithelial cell junctions.

Plain Language Summary: Microscopic fungi and fungus-like organisms that cause plant diseases often found in human environments are plant parasites. They negatively impact crop production, reduce the quality of agricultural goods, and lessen the visual appeal of decorative plants. Additionally, these fungi can pose a health hazard to humans, as they are significant sources of allergens. The most prominent allergenic fungi include *Alternaria, Aspergillus, Cladosporium, Penicillium*, and *Fusarium*. Even fungus-like organisms such as *P. ficariae*, which infects *Ficaria verna*, can provoke allergic responses. *P. ficariae* infest plants extensively, releasing large quantities of spores, especially during spring. This increases the likelihood of human exposure to allergens as the plants and their parasitic fungi proliferate in early spring. Since conventional skin or blood tests do not always identify the underlying causes of allergies, discovering new fungal allergens is essential for more accurate hypersensitivity diagnoses. Our research shows that the parasitic *P. ficariae* fungi have strong pro-inflammatory properties, which form the basis of allergenic reactions in in vitro models of the upper and lower respiratory tracts.

Keywords: phytopathogenic microfungi, *Peronospora ficariae*, *Wilsoniana bliti*, airway epithelial cells, inflammatory response

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Introduction

The incidence of allergic conditions is swiftly rising globally, with the World Allergy Organization (WAO) estimating that it ranges from 10% to 40%, depending on the nation. In many industrialized countries, allergies impact over 20% of the population.[1,2] Although allergies were once considered uncommon at the start of the 20th century, their prevalence has surged significantly in recent decades. Research from the European Academy of Allergy & Clinical Immunology (EAACI) indicates that over 150 million Europeans are currently afflicted with chronic allergic conditions, with 20% experiencing severe and debilitating forms. It is projected that half of the European population will have been affected by allergies by 2025.[3] These statistics are likely underestimated, as numerous individuals (up to 45%) do not report symptoms or receive incorrect diagnoses.[4]

The World Health Organization and the International Union of Immunological Societies (WHO/IUIS) have identified 750 allergens, including fungal ones.[5] Although there is substantial understanding of the role of fungi in the pathophysiology of allergic diseases, they are still not adequately recognized as allergens in both basic research and clinical settings. This issue is underscored by the fact that respiratory allergies to fungi affect an estimated 20–30% of atopic individuals and up to 6% of the general population.[6] Additionally, compared to pollen, fungi are more frequently responsible for allergic reactions in the lower respiratory tract.[7]

Phytopathogenic microfungi, which act as plant parasites, are frequently found in areas where humans reside. These fungi cause extensive plant infestations, leading to lower crop yields, decreased quality of plant products, and reduced aesthetic appeal of ornamental plants. The physiology and distribution of plants and fungi, along with the production of pollen and spores, are affected by such factors as geographical location, air quality, human activities, and local vegetation sources. Climate-related factors such as temperature, humidity, and extreme weather significantly influence these processes and promote the spread of these fungal species worldwide and contact with humans.[8,9]

Conventional skin or blood tests often fail to pinpoint the exact allergen causing an allergic reaction. Although the most notable allergenic fungi are generally found in the genera *Alternaria, Aspergillus, Cladosporium, Penicillium*, and *Fusarium*,[7,10] it is conceivable that the widespread native and invasive phytopathogenic microfungi, which are responsible for extensive plant infestations, might also serve as allergen sources. Therefore, we hypothesize that common phytopathogenic microfungi, which humans can inhale, can potentially trigger an allergic response.

In this study, we have detailed the genetic identification and examined the proinflammatory characteristics of two prevalent fungus-like organisms: *Peronospora ficariae*, which parasitizes *Ficaria verna*, and *Wilsoniana bliti*, a parasite of *Amaranthus retroflexus*. These fungus-like organisms have not been studied before for their ability to induce pro-inflammatory and pro-allergic reactions. By employing two cell model lines representing the upper (BEAS-2B) and lower (A549) respiratory tract cells, we illustrate the proinflammatory activity primarily of *P. ficariae* and its capacity to compromise the epithelial barrier function in lung and bronchial cells. These mechanisms are crucial to the onset of allergies and asthma. Consequently, our research significantly advances the understanding of potential new fungal allergens.

Materials and Methods

Plant Material and Morphological Identification

Our decision to concentrate on *P. ficariae* and *W. bliti* was influenced by several factors: (i) Widespread occurrence in nature − both species were found to be relatively abundant and consistently present in our environment. Their frequent detection suggests a potentially significant role in the local fungal community; (ii) Limited existing data indicate that these fungi are relatively understudied, especially regarding their potential allergenic properties. This gap in the literature makes them compelling candidates for further investigation, as they could signify previously unrecognized contributors to fungal exposure; (iii) Preliminary screening − in our initial screening, extracts from these fungi demonstrated immunoreactivity in assays utilizing sera from sensitized individuals, indicating potential allergenic properties. While further validation is ongoing, this preliminary evidence affirms their significance; (iv) Scientific novelty − considering the limited allergen characterization for these genera and the phylum Oomycota, we recognized an opportunity to provide novel data that could enhance the current understanding of fungal allergens beyond the commonly studied species.

Additionally, the hosts of the above fungi are shared in the human environment:

1) *Ficaria verna* is a common and well-documented species within its native Eurasian range (Europe, North Africa, Western Asia) and has established itself as a naturalized (and often invasive) species in North America, with additional introductions in Australasia. GBIF data indicate the strongest occurrence in Europe, Central Asia, and North America (www.gbif.org). *Peronospora ficariae* is a well-established and prevalent downy mildew pathogen of *F. verna*, particularly throughout Europe.

(2) *Amaranthus retroflexus* is globally ubiquitous, initially native to North America but now a cosmopolitan weed with hundreds of thousands of occurrence records across Europe, North Africa, Asia (eg China, Japan), South America, Africa, Australia, and New Zealand (www.gbif.org). *Wilsoniana bliti* (syn. *W. amaranthi, Albugo bliti*) is a widely distributed parasite of *Amaranthus retroflexus*, occurring in Europe, Asia, and likely worldwide, following the distribution of its host.

The fungi chosen for this study are biotrophic organisms that cannot be cultivated on synthetic substrates in a laboratory setting. Consequently, the research specimens were sourced from their natural habitats. Plant parts affected by fungus-like species from the Peronosporales (*Peronospora ficariae*) and Albuginales (*Wilsoniana bliti*) orders were gathered in Puławy and Lublin, Poland. Specifically, *P. ficariae* was found on *Ficaria verna* Huds. between April 20–24 and April 21–27, 2021 (LBL M–033124, collected by botanist and mycologist U. Świderska), and *W. bliti* was collected from *Amaranthus retroflexus* L. in Lublin on July 16, 2021, and August 10, 2021 (LBL M–033125, collected by U. Świderska). The host plant *Ficaria verna* Huds. grows most often in deciduous forests (especially riparian and oak-hornbeam forests), wet meadows, and thickets, but often in parks and gardens as a weed, while *Amaranthus retroflexus* L. grows in ruderal and segetal habitats. These plants are not protected under species conservation laws and do not reside in designated protected areas.

The gathered materials, comprising leaves and infecting fungi, were air-dried and preserved in the herbarium at Maria Curie-Skłodowska University in Lublin.[7] Initially, the botanist and mycologist U. Świderska assessed the specimens' morphological traits using microscopic slides stained with Lactophenol Cotton Blue dye. Observations were made with an Olympus BX53 light microscope at magnifications of 40x, 100x, 400x, and 600x. Microphotographs of the diagnostic structures of the fungi were taken with an Olympus digital camera SC180 attached to the microscope and an Olympus SZ10 stereoscopic microscope equipped with an Olympus camera XC50. Subsequently, the structures were coated with gold using an Emitech K550X Sputter Coater and examined with a TESCAN Vega 3 LMU scanning electron microscope from Brno, The Czech Republic. Specimens displaying fungal morphological features, such as conidia and conidiophores, were then prepared for further laboratory analysis under the guidance of an Olympus SZ61 stereoscopic microscope. The samples were placed in test tubes and exposed to liquid nitrogen vapor for 24 hours before grinding into a powder with a mortar and pestle. This powdered fungal material was utilized to create crude extracts.

Genetic Identification of Fungus-Like Species

DNA Extraction

DNA was extracted from the fungal cells using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The genomic DNA's purity and concentration were assessed using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). The DNA was stored at –20°C.

Genetic Analysis

The taxonomic position of the tested isolates was determined by comparative analysis of the internal transcribed spacer (ITS) region, the large subunit (LSU) sequence of the rRNA gene, and the *cox1* and *cox2* gene sequences. These sequences were amplified using primer pairs ITS5-u/ITS4-u and LR0R/LR6, as described by Vilgalys and Hester[11] and Pfunder et al.[12] The *cox1* and *cox2* genes were amplified using the primers and conditions described by Robideau et al[13] and Hudspeth et al.[14]

According to the manufacturer's guidelines, the PCR amplifications were carried out with the ReadyMix Taq PCR Reaction Mix kit (Sigma-Aldrich). Each reaction mixture included 50–100 ng of template DNA and 0.4 mM of each primer (Table S1). Following established protocols, the PCR reactions were executed in a thermocycler (TProfessional BASIC 96 Gradient, Biometra GmBH, Göttingen, Germany).[15]

The amplicons obtained were purified using a Clean-Up purification kit from A&A Biotechnology and then sequenced with a Terminator Cycle sequencing kit. Sequencing was conducted by Genomed (Warsaw, Poland). The sequences obtained were compared to those available in the GenBank database using the BLAST tool. Sequence alignments were carried out with ClustalX2[16] and analyzed using the GeneDoc program.[17] A phylogenetic tree was created from the sequences using the Neighbor-Joining (NJ) method in the MEGA11 program,[18] employing the Kimura two-parameter model for nucleotide substitution. The most suitable evolutionary model for each gene was determined using jModelTest.[19] A bootstrap test with 1000 replicates evaluated the statistical significance of the tree. The phylogenetic tree was visualized with the TreeView program.[20]

The GenBank Accession Numbers

The GenBank accession numbers of the analyzed sequences are as follows: LSU: OQ606397, ITS: OQ606396, COI OQ623382, COII: OQ623383 for *Peronospora ficariae* and LSU: OQ613350, ITS: OQ606412, COI: OQ623380, COII: OQ623381 for *Wilsoniana bliti*. The accession numbers of the reference strains are shown in the phylograms.

Preparation of Crude Fungal Extracts

The fungal biomass underwent three acetone rinses and was then dried for a full day at 37°C. Once dried, the material was mixed with 0.05M Tris-HCl buffer at pH 8.0, using a ratio of 1 mL for every 10 mg of dry weight. This mixture was subjected to three sonication cycles, each consisting of 20 seconds of sonication followed by a 2-minute cooling period in a water bath at room temperature (Elmasonic S100H, Elma, Germany). The extraction process took place overnight with shaking at 4°C, after which the extracts were centrifuged at 804 × g for 10 minutes at 4°C. The supernatants were transferred into a regenerated cellulose membrane with a molecular weight cut-off of 6–8 kDa (Spectrum Laboratories, Rancho Dominguez, CA, USA) and dialyzed for 24 hours at 4°C against 0.1M NH_4HCO_3 buffer at pH 8.4, with the buffer replaced three times. The material was subsequently lyophilized and reconstituted in phosphate-buffered saline (PBS, Biomed, Poland). The protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a standard.[21] The crude fungal extracts prepared in three separate replicates were divided into 100 µL portions and stored at −80°C for future analysis.

Cell Lines and Culture Media

In the in vitro studies, two types of adherent human airway cell lines were used: human alveolar epithelial cancer cells (A549, ATCC# CCL-185) and human normal bronchial epithelial cells (BEAS-2B, ATCC# CRL-9609). These cell lines are widely accepted models of the upper (BEAS-2B cell line) and lower (A549 cell line) respiratory tracts in in vitro studies related to allergy response.[22–24] The A549 cells were grown in a 1:1 (v/v) combination of RPMI 1640 (Corning Media) and DMEM (Sigma-Aldrich) enriched with 10% (v/v) heat-inactivated fetal bovine serum (FBS, EURx Molecular Biology Products) and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich). Initially, the BEAS-2B cells were cultured in LHC-8 medium (Gibco) containing 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin; after 24 hours, the medium was switched to serum-free LHC-8 to preserve the typical cuboidal, polygonal shape of respiratory epithelium cells.[25] Both cell lines were maintained in standard conditions at 37°C, with 5% CO2 and 95% humidity. The cells were cultured until they reached confluence and then passaged at a 1:3 ratio using PBS without Ca^{2+} and Mg^{2+} ions and 0.25% trypsin with 0.02% EDTA (Biological Industries). Cell line studies were performed in a Safety Level 2 (BSL2) laboratory.

Cytotoxicity Study with the MTT Assay

Cell viability was determined by measuring mitochondrial activity, which was assessed through the conversion of the yellow tetrazolium salt, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), into purple formazan crystals. The color intensity, analyzed spectrophotometrically from dissolved crystals, reflected the mitochondrial activity in live cells.[26] In summary, the cells were plated in 96-well plates (100 µL per well) at a concentration of 1×10^5 cells/mL for the A549 cell line or 2×10^5 cells/mL for the BEAS-2B cell line, using an appropriate culture medium with 10% FBS (Nunc, Roskilde, Denmark), and incubated at 37°C for 24 hours. After the medium was removed, 100 µL of fungal extracts at different concentrations (0.09–400 µg of protein/mL) in the suitable culture medium (RPMI 1640 + DMEM

with 2% FBS for A549 cells; serum-free LHC-8 for BEAS-2B cells) were added to the wells. The cells were then incubated again at 37°C for another 24 hours. Controls included untreated cells and medium alone, with blank wells prepared without cells. The MTT assay was conducted as outlined in previous research.[27] Optical absorbance at 570 nm (A570) was measured using a VICTOR X4 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). The results were expressed as (i) the percentage of cell viability compared to the control and (ii) IC50, which is the concentration needed to achieve a 50% reduction in cell viability. Each experiment was performed in independent triplicate with 4–8 samples per run. The percentage of viable cells was calculated using the following formula:

Cell viability (%) = (A570 of treated cells – A570 of blank/A570 of control cells – A570 of blank) × 100, where A570 represents absorbance measured at a wavelength of 570 nanometers (nm).

Flow Cytometry

Cytotoxicity Study

The cells were introduced into 24-well plates, with each well receiving 1 mL, at a concentration of 1×10^5 cells/mL for the A549 cell line and 2×10^5 cells/mL for the BEAS-2B cell line (Nunc, Roskilde, Denmark). They were cultured in the appropriate medium containing 10% FBS and incubated at 37°C for 24 hours. Following the removal of the medium, fresh medium was added along with the fungal extracts to be evaluated, with concentrations determined by the MTT assay results (RPMI 1640 + DMEM with 2% FBS for the A549 cells and serum-free LHC-8 for the BEAS-2B cells). Untreated cells served as the negative control, while cells treated with 5% DMSO (Sigma-Aldrich) were used as the positive control. After a 24-hour incubation period, supernatants were collected from each well and transferred to cytometry tubes. Subsequently, 150 µL of accutase (Corning Media) was added to each well to detach the cells. The contents of each well were resuspended in 500 µL of the respective culture medium (RPMI 1640 + DMEM with 2% FBS for the A549 cells and serum-free LHC-8 for the BEAS-2B cells), transferred to cytometry tubes containing the corresponding supernatants, and centrifuged at 314 × g for 5 minutes at room temperature. The supernatants were discarded, and the cells were washed with 500 µL of binding buffer (10X Annexin V Binding Buffer, diluted 1:9 in H2O, BD Biosciences). After another centrifugation at 314 × g for 5 minutes at room temperature, the cell pellet was resuspended in 100 µL of binding buffer with 5 µL of annexin V FITC (BD Biosciences) and 5 µL of propidium iodide (BD Biosciences), gently vortexed, and incubated for 15 minutes at room temperature, shielded from light. Finally, 400 µL of binding buffer was added to each cytometry tube, and the samples were analyzed using a flow cytometer (FACSCalibur™ Flow Cytometer, BD Biosciences) with Cell Quest Pro software. Each experiment was conducted in triplicate. The results are expressed as the percentages of viable, apoptotic (early and late), and necrotic cells among the total cells analyzed. A representative flow cytometry gating strategy for this analysis is presented in Figure 1.

Determination of Reactive Oxygen Species Production

Cells from both cell lines were prepared in a PBS solution lacking Ca^{2+} and Mg^{2+} ions, with 1% FBS added, at a concentration of 2×10^5 cells/mL. A volume of one milliliter of this cell suspension was transferred into a cytometric tube, followed by the addition of 1 µL of dihydrorhodamine 123 (DHR 123, Thermo Fisher Scientific) to achieve a final concentration of 5 mM/mL in PBS without Ca^{2+} and Mg^{2+}. The mixture was then incubated at 37°C for 15 minutes. Fungal extracts were introduced into the cytometric tubes at concentrations determined by the MTT assay results.

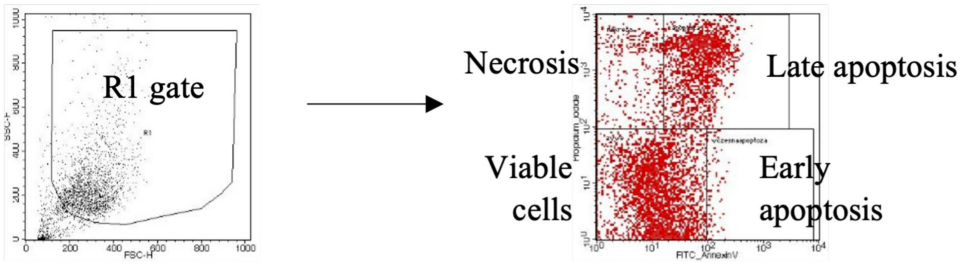

Figure 1 A representative flow cytometry gating strategy for cytotoxicity analysis.

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Untreated cells were used as a negative control, while cells treated with 5 µL of tert-butyl hydroperoxide (tBHP, Sigma-Aldrich) at a final concentration of 50 µM in PBS served as a positive control. After 1-hour incubation at 37°C, the tubes were centrifuged at 314 × g for 5 minutes at room temperature, and the supernatant was removed. The cell pellet was resuspended in 500 µL of PBS with 1% FBS, and flow cytometric analysis was conducted using a FACSCalibur™ Flow Cytometer (BD Biosciences) and Cell Quest Pro software. Based on three independent experiments, the results were expressed as the percentage of cells generating reactive oxygen species compared to those that did not. A representative flow cytometry gating strategy for this analysis is shown in Figure 2.

Determination of Cytokine Production

In our study, the capacity of the fungal extract to enhance the production of IL-1β, IL-6, TNF-α, TGF-β, and GM-CSF in A549 and BEAS-2B cells was assessed. We seeded A549 cells at a density of 1×10^5 cells/mL and BEAS-2B cells at 2×10□ cells/mL into 24-well plates (1 mL per well) (Nunc, Roskilde, Denmark) using a suitable culture medium with 10% FBS, and incubated them at 37°C for 24 hours. After discarding the old medium, fresh medium and fungal extracts were introduced: RPMI 1640 + DMEM with 2% FBS for the A549 cells and serum-free LHC-8 for the BEAS-2B cells. The concentration of fungal extracts was determined based on the MTT assay results: (i) a concentration equal to 50% of the IC50 value and (ii) a concentration where cell viability was between 80–90%. Untreated cells were used as a negative control. Each experiment was conducted in triplicate. Following 24-hour incubation at 37°C, culture supernatants were collected and centrifuged (314 × g, 5 minutes, room temperature). The samples were then stored at −70°C for later analysis. Enzyme-linked immunosorbent assays (ELISA, Shanghai Coon Koon Biotech Co.) were performed according to the manufacturer's guidelines. The ELISA sensitivity for each cytokine was as follows: IL-1β 10.0 pg/mL, IL-6 1.0 pg/mL, TNF-α 10.0 pg/mL, TGF-β 10.0 pg/mL, and GM-CSF 10.0 pg/mL.

Determination of the Presence of E-Cadherin and Occludin with Fluorescence Microscopy

The A549 cell line was seeded at a density of 3×10^5 cells/mL, while the BEAS-2B cell line was seeded at 6×10^4 cells/mL in suitable culture media into 8-chamber slides, with each chamber receiving 0.5 mL (Thermo Fisher Scientific). The cells were incubated at 37°C for 24 hours. Subsequently, the medium containing 10% FBS was replaced with fresh medium containing fungal extracts in the same concentration range used for cytokine analysis: RPMI 1640 + DMEM with 2% FBS for the A549 cells and serum-free LHC-8 for the BEAS-2B cells. After incubation for another 24 hours at 37°C, the supernatant was discarded, and the chambers were washed three times with PBS containing Ca^{2+} and Mg^{2+} ions (500 µL per chamber). The cells were then fixed with 4% paraformaldehyde (400 µL per chamber, BD Biosciences) for 15 minutes at 37°C, followed by three washes with 500 µL of PBS. To permeabilize the cell membranes, 400 µL of 0.1% Triton X-100 (Sigma-Aldrich) in PBS was added to each chamber and incubated for 15 minutes at room temperature. After another three washes with PBS, 500 µL of 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS was added to each chamber to block non-specific antibody binding sites. The chambers were incubated for 60 minutes at room temperature before the supernatant was removed, and 100 µL per well of primary antibody (occludin - 5 µg/mL in 2% BSA, mouse/IgG1, kappa, clone OC-3F10; E-cadherin - 2 µg/mL in 2% BSA, mouse/IgG1, clone HECD-1) was added (Thermo Fisher Scientific). Following overnight incubation at 4°C, the chambers were rinsed three times with 500 µL of

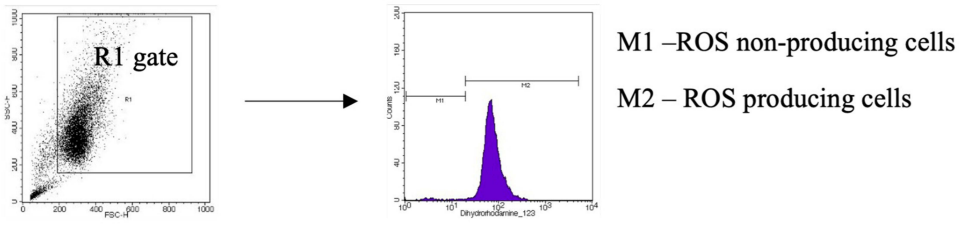

Figure 2 A representative flow cytometry gating strategy for reactive oxygen species production analysis.

PBS, and 100 µL per well of Alexa Fluor™ 488-conjugated secondary antibody (2 µg/mL in 2% BSA, goat polyclonal anti-mouse IgG) was added (Thermo Fisher Scientific). The slides were incubated for 45 minutes at room temperature in the dark. After removing the supernatant, 200 µL of DAPI (2 µg/mL in PBS) (Sigma-Aldrich) was added to each chamber and incubated in the dark for 5 minutes at room temperature. The chamber walls were removed, the slides were rinsed with PBS, and a few drops of a glycerol/water solution (1:1, v/v) were applied. The slides were covered with coverslips, and images were captured using a DM4000B epifluorescence microscope (Leica, Wetzlar, Germany) equipped with Leica EL6000 filters, a Leica DFC 500 camera, and LAS V3.1 Leica image analysis software.

Additional control samples were created using cells that were not exposed to fungal extracts: (i) cells lacking primary antibodies and (ii) cells that were only stained with secondary antibodies linked to a fluorescent dye to verify the specificity of the fluorescent staining.

Statistical Analysis

Continuous variables that followed a normal distribution were presented as mean ± SD based on a minimum of three separate experiments. The Mann–Whitney *U*-test or one-way ANOVA, followed by Tukey's post-hoc multiple comparison test, was used to assess statistical significance between groups. Statistical analysis was performed using STATISTICA version 12 (StatSoft, Inc., Tulsa, OK, USA), with a P value of <0.05 deemed significant. The IC50 (inhibitory concentration) was determined using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Plant Material and Morphological Identification of Fungus-Like Species

Symptoms of *Peronospora ficariae* infection were visible on the lower surface of *Ficaria verna* leaves in the form of a dense gray-purple coating consisting of conidiophores and conidia (Figure 1A: a and b). Conidiophores of *P. ficariae* measured 226 to 317 µm in length (average = 270 µm, n = 30) and 6.6 to 10 µm in width (average = 8.24 µm, n = 30). Conidia measured 22.8 to 27.8 µm in length (average = 25.38 µm, n = 30) and 17.0 to 23.5 µm in width (average = 20.12 µm, n = 30) (Figure 3A: c-f). Symptoms of *Wilsoniana bliti* infection appeared as light-green chlorotic spots on the upper surface of the *Amaranthus retroflexus* leaves, while the blister sori formed on the corresponding lower surface was white-yellow, oval to ellipsoidal (Figure 3B: a-c). The cylindrical conidiophores measured 25–55 × 10-14 µm. *W. bliti* produced conidiophores measuring 24–56 × 3.7–8.7 µm (average = 56 × 11.1 µm, n = 30), and conidia measuring 16.8–21.0 × 11.8–17.6 µm (average = 17.8 × 16.1 µm, n = 30) (Figure 3B: e-f).

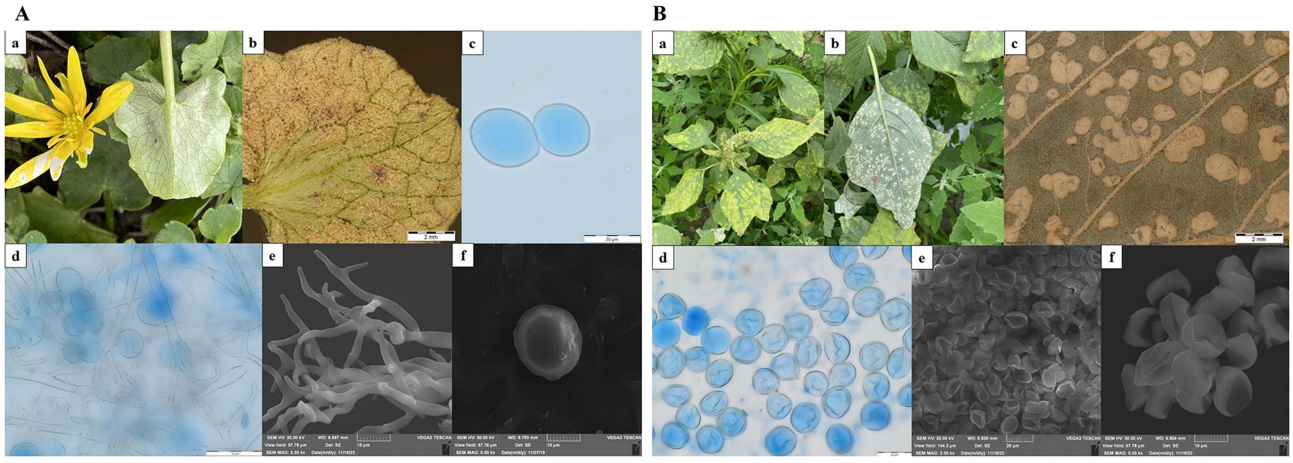

Figure 3 (**A**) *Peronospora ficariae* on *Ficaria verna*. a – disease symptom on the underside of infected leaves; b – lower surface of the leaf with abundant sporulation with densely packed conidiophores; c – conidia; d – conidiophores and conidia; e – apical branches of conidiophores; f – conidium. Scale: b – 2 mm; c – 20 µm; d – 50 µm; e, f – 10 µm. (**B**) *Wilsoniana bliti* on *Amaranthus retroflexus*. a, b – disease symptoms on the upper and lower sides of infected leaves; c – white blister rust on the underside of the leaf; d, e, f – conidia. Scale: c – 2 mm; d, e – 20 µm; f – 10 µm.
Abbreviations: SM, stereo microscope; LM, light microscope; SEM, scanning electron microscope.

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The morphological characteristics of *P. ficariae* and *W. bliti* align with the species descriptions provided by Kochman & Majewski.[28]

Phylogenetic Analysis

The taxonomic position and phylogenetic relationships of the fungal isolate from *Ficaria verna* were determined by comparing the concatenated sequences of the LSU and ITS regions to the corresponding sequences of reference strains from the GenBank database. The studied strain showed the most excellent phylogenetic relationship to representatives of the genus *Peronospora* (from 94% to 98% similarity of the analyzed LSU+ITS sequences). In the phylogenetic tree constructed for the concatenated LSU+ITS sequences, the isolate from *Ficaria verna* formed a typical cluster with strains representing the species *Peronospora ficariae* (with bootstrap support of 100%) (Figure 4A). In addition to the analysis of ITS and LSU sequences, other genetic markers are also increasingly being used for the identification, classification, and phylogenesis of various groups of oomycetes, eg genes encoding cytochrome c oxidase subunits 1 and 2 (*cox1* and *cox2*).[29] The comparative analysis of the sequences of the *cox1* and *cox2* marker genes of the tested isolate with the corresponding sequences of these genes available in the GenBank database confirmed its taxonomic affiliation to the fungi of the genus *Peronospora* (from 90 to 100% sequence similarity). In the phylogenetic trees created for the *cox1* and *cox2* genes, the *Ficaria verna* isolate formed a typical cluster with *P. ficariae* strains (bootstrap value 100%) (Figure 4B and C).

The comparative sequence analysis of the LSU region performed using the NJ method showed that the isolate from *Amaranthus retroflexus* represents a fungus-like oomycete (Oomycota) of the genus *Wilsoniana* (from 90% to 99% sequence similarity). The studied strain showed the highest sequence similarity to the corresponding sequences of strains of *Wilsoniana amaranthi* (99%). In the phylogenetic tree of the LSU region, species of the genus *Wilsoniana* formed a common cluster with a bootstrap value of 100% (Figure 5A). According to the Index Fungorum database [https://www.indexfungorum.org], *W. amaranthi* is currently considered a synonym of *W. bliti* (Figure 5A).

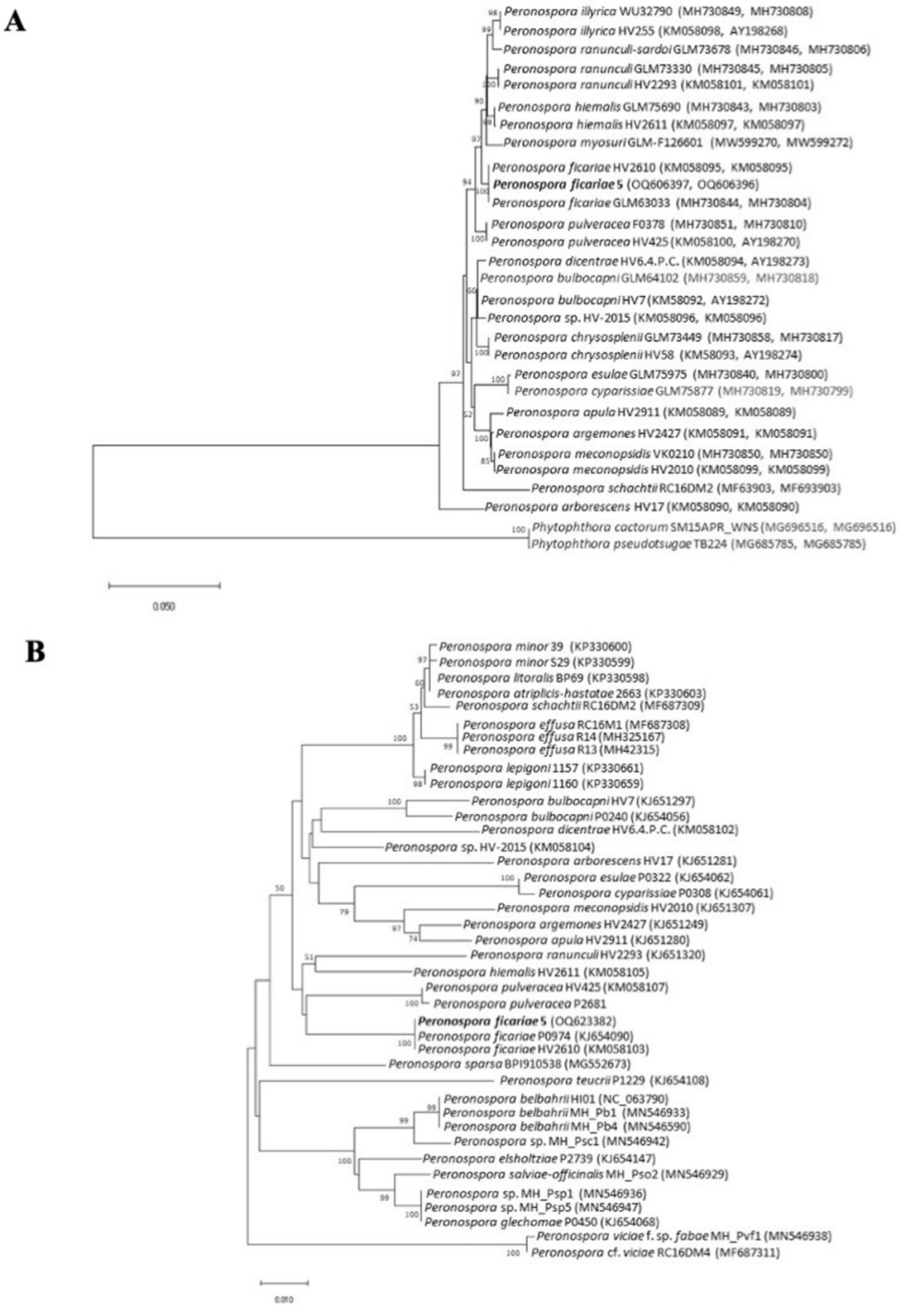
In the phylogenetic tree of the ITS region, the isolate from *A. retroflexus* grouped with the strain representing the species *W. amaranthi* (bootstrap value 100%) (Figure 5B). The similarity of the ITS sequence of the isolate to the sequence of the reference strain *Wilsoniana amaranthi* was 96%. The degree of similarity of the sequence of the tested strain to the sequences of other representatives of the genus *Wilsoniana* ranged from 80% to 83%. The phylogenetic analysis based on the *cox1* and *cox2* genes confirmed the affiliation of the isolate from A. *retroflexus* to the genus *Wilsoniana*. In the phylogenetic trees of both these genes, the isolate from *A. retroflexus* formed a common group with strains representing the species *W. amaranthi* (Figure 5C and D).

Characteristics of Biological Properties of Peronospora ficariae and Wilsoniana bliti

Cytotoxicity Study (MTT Assay and Flow Cytometry)

The bronchial epithelial cells were found to be sensitive to the action of the *Peronospora ficariae* extract in the range of the three highest concentrations tested, ie 100 µg protein/mL, 200 µg protein/mL, and 400 µg protein/mL, and the percentage of metabolically active cells was 26.6 ± 1.2%, 7.6 ± 0.0%, and 6.6 ± 0.0%, respectively. Statistically significant changes indicating cytotoxic activity of the extract were also noted after the incubation of the alveolar epithelial cells with the extract at a concentration of 200 µg protein/mL and 400 µg protein/mL (reduction in viability by about 67–93%). In comparison with the A549 cells, the bronchial epithelial cells were more sensitive to the action of the *P. ficariae* extract, which was confirmed by the significantly lower ($P < 0.05$) percentage of viable BEAS-2 cells in the concentration range of 200–100 µg protein/mL and the IC50 parameter values of 165.3 ± 3.2 µg protein/mL and 81.8 ± 2.8 µg protein/mL for the A549 and BEAS-2B cells, respectively (Figure 6A).

In the case of the A549 cells, the 24-hour incubation with the highest fungal extract concentration (400 µg protein/mL) did not allow separation of the cells into individual subpopulations due to the strong cytotoxic effect. Compared to the negative control (without the addition of the extract), *P. ficariae* at a concentration of 200 µg protein/mL caused a significant ($P < 0.05$) increase in the percentage of early apoptotic (up to 31.2 ± 2.2%), late apoptotic (up to 40.9 ± 2.5%), and necrotic (up to 29.5 ± 1.8%) cell subpopulations (Figure 6B). The BEAS-2B cells were characterized by greater sensitivity to the fungal extract at a concentration of 100 µg protein/mL; the percentage of early and late apoptotic alveolar epithelial cells was significantly higher ($P < 0.05 – P \leq 0.001$) than that in the alveolar epithelial cells. The above



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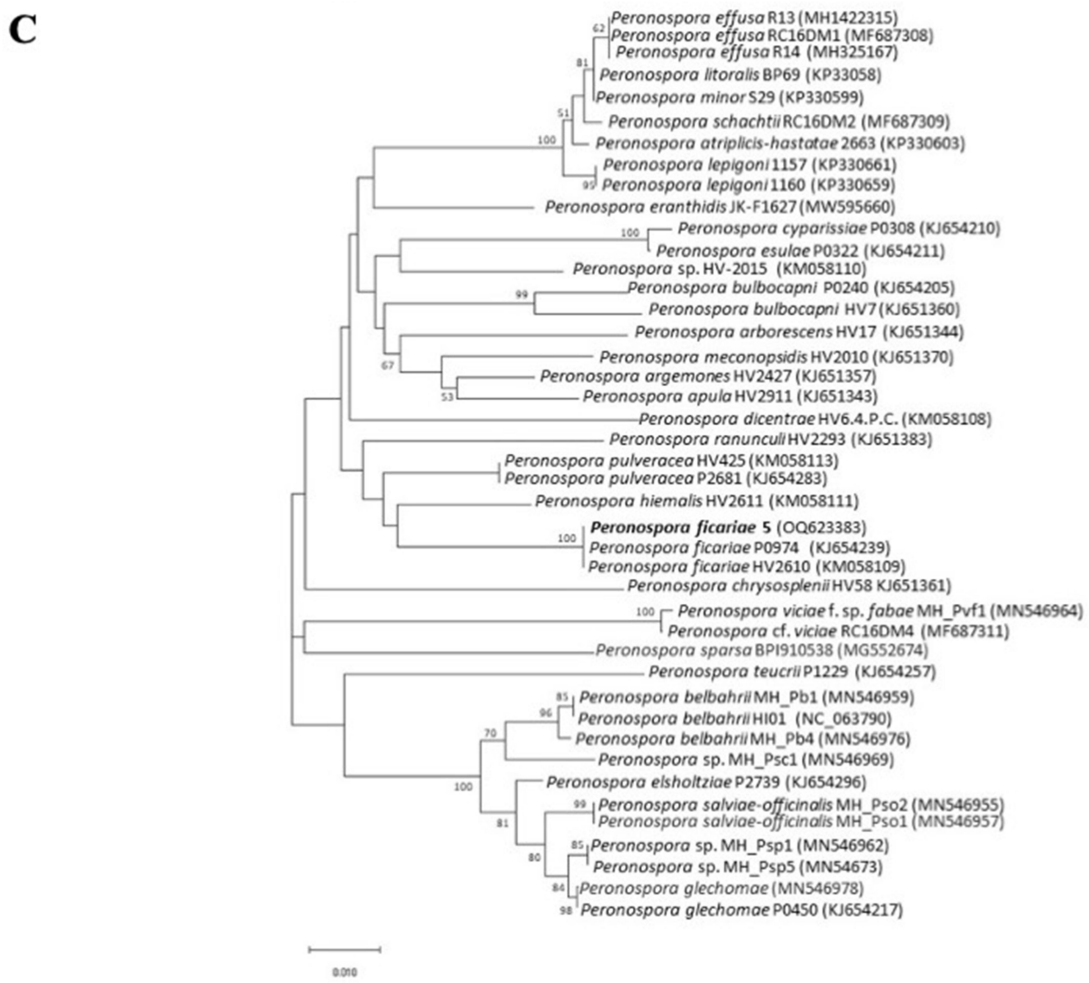


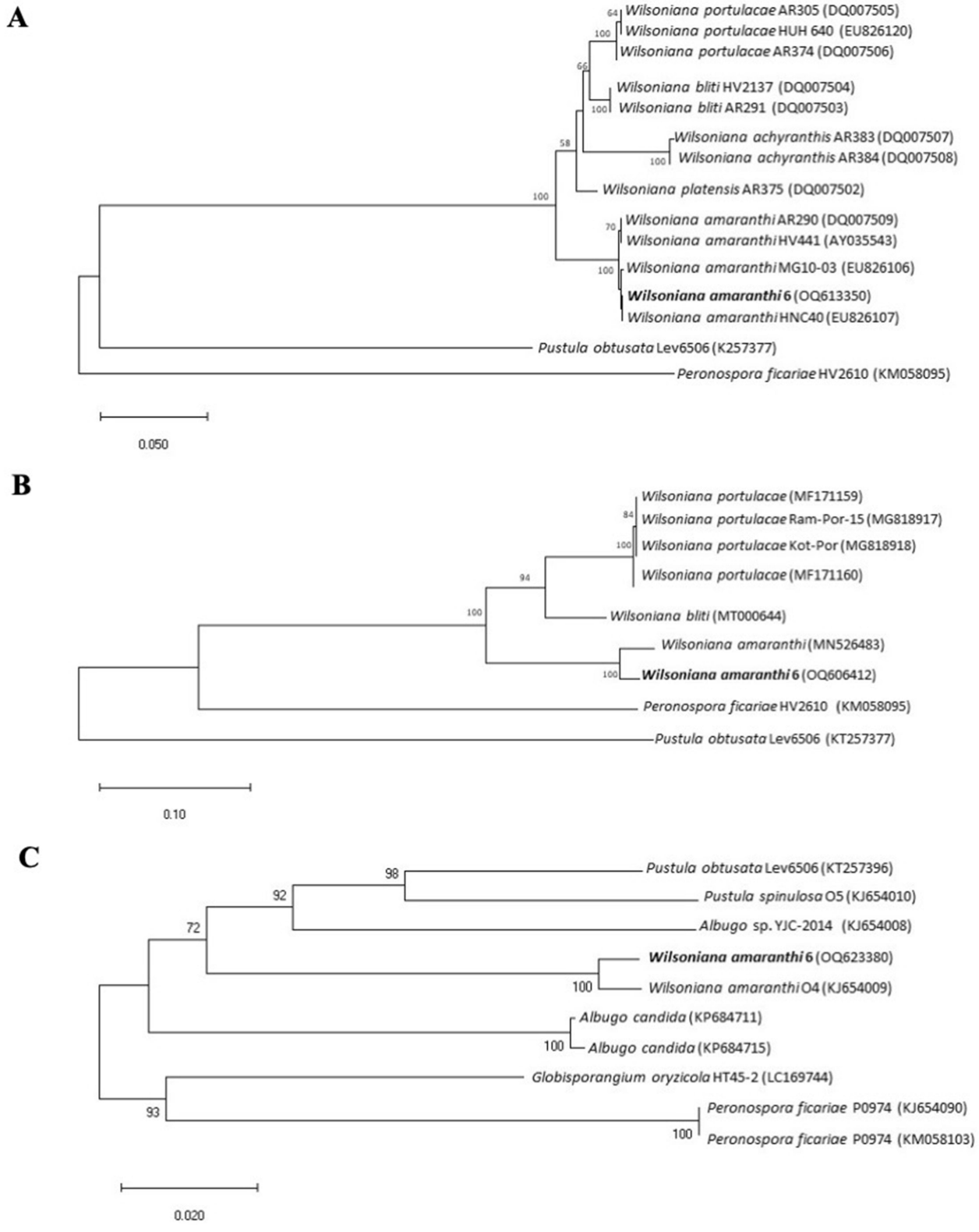
Figure 4 (**A**) Phylogenetic tree showing the phylogenetic relationships of the *Ficaria verna* isolate and reference strains from the GenBank database constructed based on the sequence similarity of the LSU + ITS regions. (**B**) Phylogenetic tree showing the phylogenetic relationships of the *Ficaria verna* isolate and reference strains from the GenBank database constructed based on *cox1* gene sequence similarity. (**C**) Phylogenetic tree showing the phylogenetic relationships of the *Ficaria verna* isolate and reference strains from the GenBank database constructed based on *cox2* gene sequence similarity. Bootstrap values (≥50) are shown on the branches. The scale bar indicates the number of substitutions per site.

cell subpopulations were substantially more numerous in the case of the BEAS-2B cells after the incubation with 100 µg protein/mL of the *P. ficariae* extract (Figure 6C).

The MTT test results showed no cytotoxic effect of the *W. bliti* extract on both A549 and BEAS-2B cell lines in the concentration range of 0.09–400 µg protein/mL, and the IC50 values for both cell lines were higher than 400 µg protein/mL (Figure 6D). The flow cytometric analysis confirmed the results of the MTT test - no differences were noted in the content of early and late apoptotic and necrotic cells A549 and BEAS-2B cells after the contact with the *W. bliti* extract, compared to the negative control (Figure 6E and F).

Determination of Reactive Oxygen Species Production (Flow Cytometry)

To determine whether reactive oxygen species (ROS) may be the probable cause of the cytotoxic effect of the fungal extract, a cytometric analysis was performed using dihydrorhodamine 123 dye. The flow cytometric analysis using the *P. ficariae* extract at the concentrations of 200 µg protein/mL and 400 µg protein/mL along with the A549 and BEAS-2B cell lines did not allow separation of subpopulations of cells that produced and did not produce ROS, likely due to the toxicity of these concentrations (Figure 7A and B). However, the extract tested at concentrations ranging from 12.5 to 100 µg protein/mL successfully induced the production of reactive oxygen species in the alveolar epithelial cells, as



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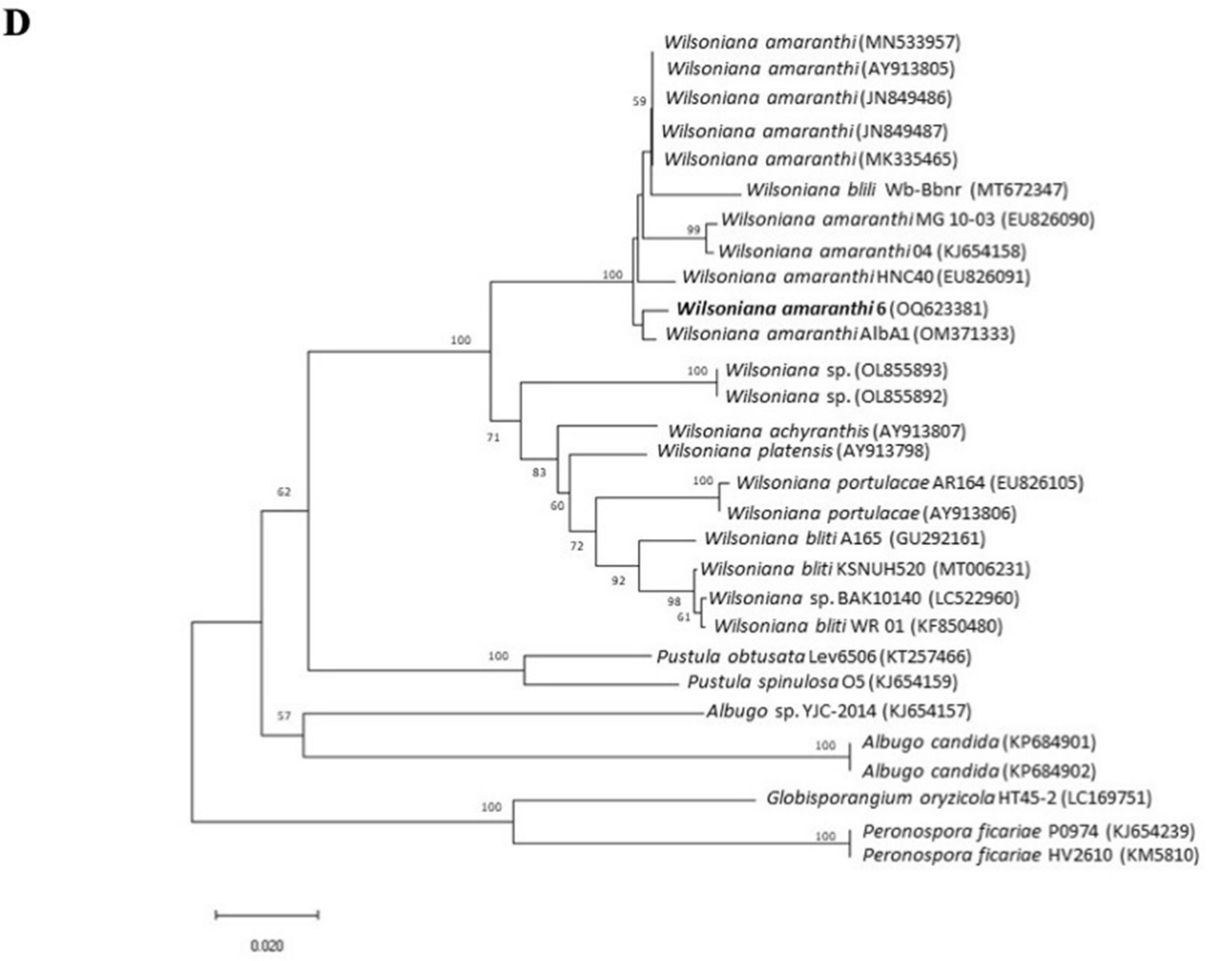

Figure 5 Phylogenetic tree showing the phylogenetic relationships of the *Amaranthus retroflexus* isolate and GenBank reference strains, constructed based on the sequence similarity of the LSU region (**A**), ITS region (**B**), the *cox1* gene (**C**), and the *cox2* gene (**D**). Bootstrap values (≥50) are shown on the branches. The scale bar indicates the number of substitutions per site (*Wilsoniana amaranthi* = *Wilsoniana bliti*).

evidenced by the higher percentage of ROS-producing cells compared to the negative control (3.7 ± 0.2%), with levels ranging from 16.7 ± 1.0% to 44.7 ± 2.1% - these difference were statistically significant ($P < 0.05$-$P \leq 0.001$). The fungal extract at the concentrations of 25 and 50 µg protein/mL induced ROS production more effectively in the A549 cells than in the BEAS-2B cells (Figure 7A and B). The *W. bliti* extract in the concentration range of 100–400 µg protein/mL did not cause ROS production in both cell lines tested (A549 and BEAS-2B) (Figure 7C and D).

Determination of Cytokine Production

Proinflammatory cytokines that are crucial in developing allergic reactions - IL-1β, IL-6, TNF-α, TGF-β, and GM-CSF - were selected for this study. Based on the results of cytotoxicity and reactive oxygen production, two concentrations of the *P. ficariae* extract were used: 83 µg protein/mL and 50 µg protein/mL for the A549 line and 41 µg protein/mL and 25 µg protein/mL for the BEAS-2B line (the principle of selecting concentrations is described in the Materials and Methods section). In the case of *W. bliti*, due to the lack of observed cytotoxicity, the highest extract concentrations were selected for both cell lines in this experiment – 200 µg protein/mL and 100 µg protein/mL.

In response to 25 µg protein/mL and 41 µg protein/mL of the *P. ficariae* extract, the BEAS-2B cells produced significantly ($P < 0.05$) more IL-1β, with values of 27.6 ± 2.4 pg/mL and 19.2 ± 1.2 pg/mL, respectively. A slightly lower yet still statistically significant increase in the IL-1β levels was observed after the contact with the A549 cells treated with

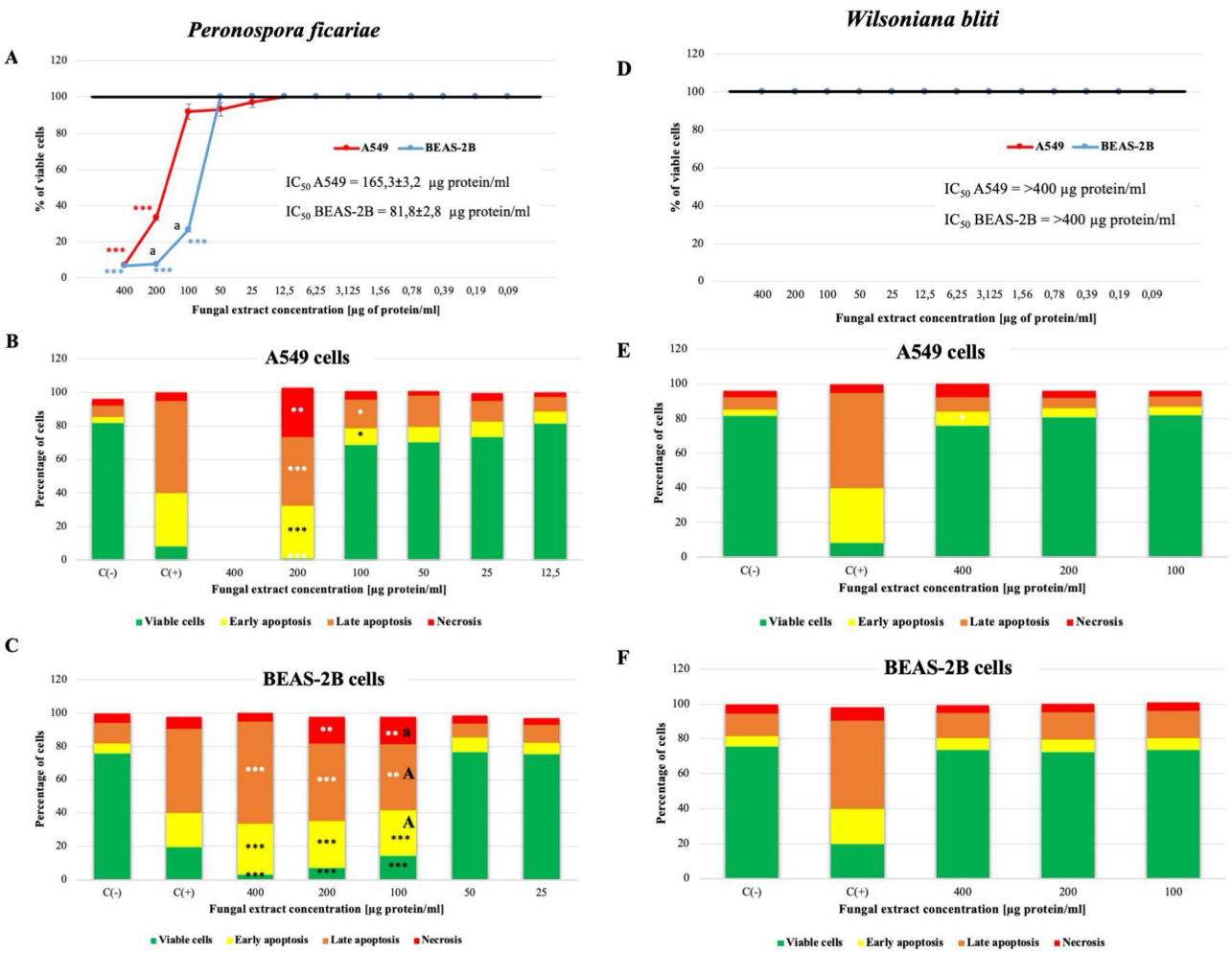

Figure 6 Evaluation of the viability of A549 and BEAS-2B cell lines after 24-hour incubation with *P. ficariae* and *W. bliti* extracts (MTT (**A** and **D**) and flow cytometry (**B, C, E** and **F**)). The results are mean values ± SD from three independent experiments (4–8 replicates). C(-) – negative control, C(+) – positive control (DMSO [5%]). * statistically significant difference compared to the control sample (Mann–Whitney *U*-test), *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001. [a] – statistically significant difference compared to the A549 cells (one-way ANOVA with Tukey's post-hoc test), [a]P < 0.05, [A]P ≤ 0.01.

50 µg protein/mL and 83 µg protein/mL of extract, resulting in values of 14.7 ± 0.3 pg/mL and 10.2 ± 0.2 pg/mL, respectively (Figure 8A and 8B). The GM-CSF concentrations of 19.3 ± 1.0 pg/mL and 15.8 ± 0.8 pg/mL indicate that it was the most abundant cytokine among all those produced by the alveolar epithelial cells induced with 50 µg protein/mL and 83 µg protein/mL of the *P. ficariae* extract, respectively. In contrast, the extract concentrations of 25 µg protein/mL and 41 µg protein/mL were weak inducers of GM-CSF production in the BEAS-2B cells, as evidenced by the lack of a statistically significant increase in the concentration of this cytokine (Figure 8A and B). Overall, the ELISA tests did not reveal increased secretion of IL-6, TNF-α, and TGF-β by the alveolar and bronchial epithelial cells at any concentrations of the *P. ficariae* extract tested.

The experiment showed that, after the incubation of the BEAS-2B cells with the lower concentration of *W. bliti* extract (100 µg protein/mL), the concentration of GM-CSF and IL-1β was 22.5 ± 1.6 pg/mL and 18.7 ± 1.2 pg/mL, respectively (statistically significant values at P < 0.05) (Figure 8C and D). Considering the higher concentration of the fungal extract (200 µg protein/mL), an almost four-fold lower level of IL-1β and a five-fold decrease in the concentration of GM-CSF were noted, compared to the concentration of 100 µg protein/mL. In contrast to the bronchial epithelial cells, no increased production of IL-1β and GM-CSF was observed in the A549 cells, regardless of the concentration of the fungal extract used (Figure 8C and D). Moreover, the *W. bliti* extract did not induce the production of cytokines IL-6, TNF-α, and TGF-β (regardless of the type of cell line and extract concentration).

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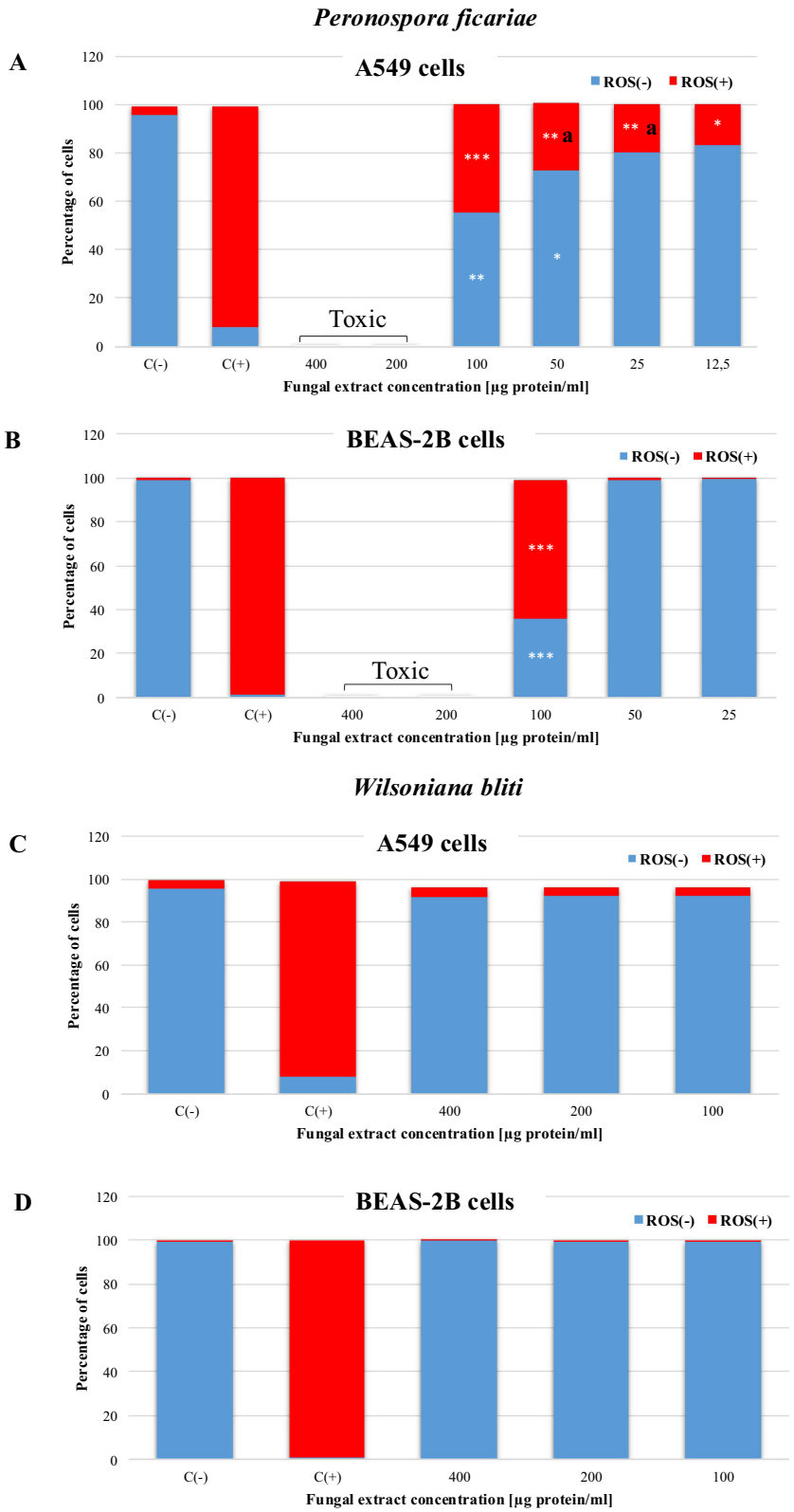

Figure 7 Assessment of ROS production by A549 and BEAS-2B cells after 24-hour incubation with *P. ficariae* (**A** and **B**) and *W. bliti* fungal extracts (**C** and **D**). The results are mean values ± SD from three independent experiments. C(-) – negative control (without added fungal extract), C(+) – positive control (tBHP [50µM]), ROS(-) – ROS non-producing cells, ROS(+) – ROS producing cells. * statistically significant difference in comparison with the negative control (Mann–Whitney *U*-test), *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001. [a] – statistically significant difference in comparison with the BEAS-2B cells (one-way ANOVA with Tukey's post-hoc test), [a]P < 0.05.

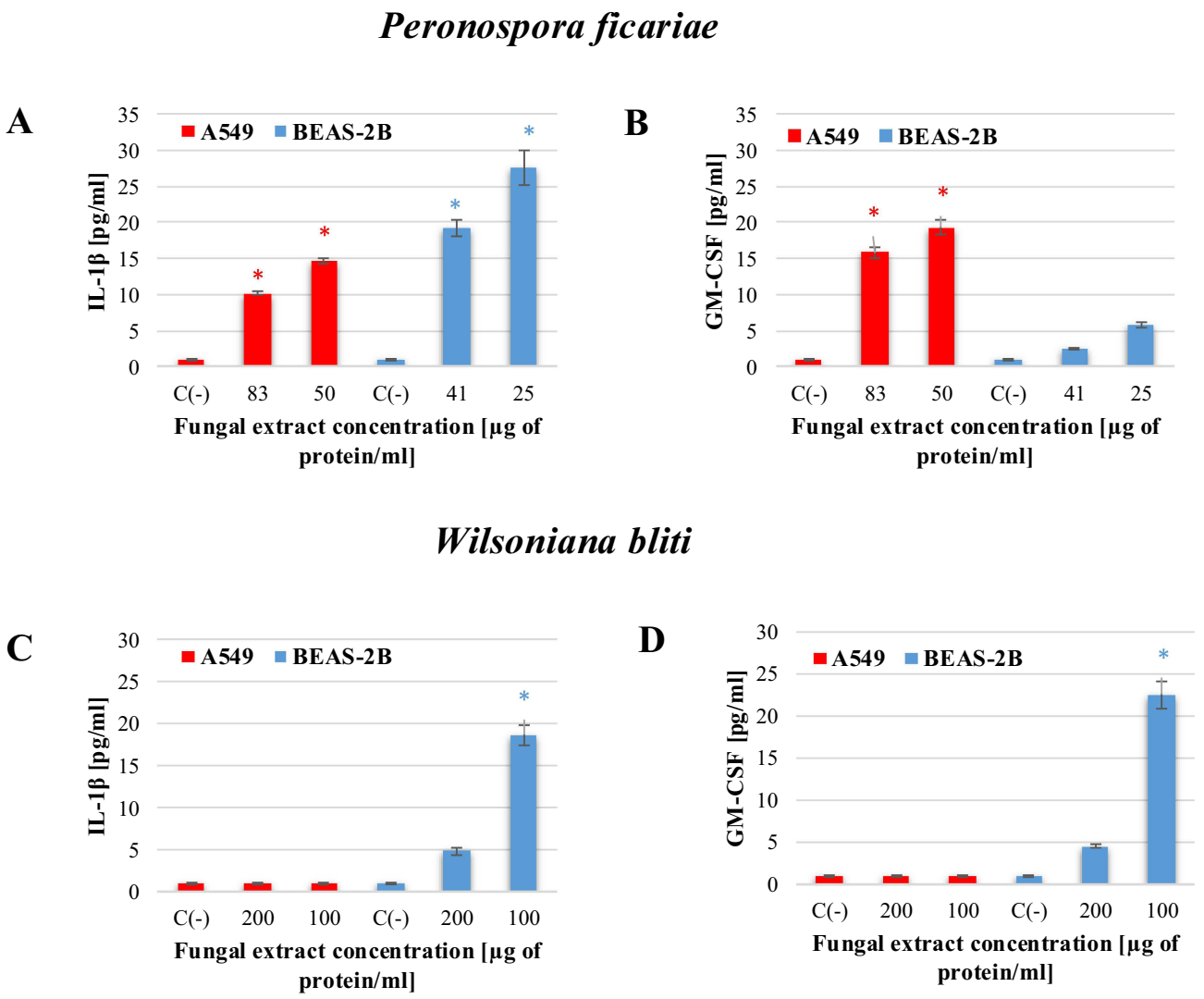

Figure 8 Assessment of IL-1β and GM-CSF production by A549 and BEAS-2B cells after 24-hour incubation with *P. ficariae* (**A** and **B**) and *W. bliti* (**C** and **D**) fungal extracts. The results are mean ± SD values from three independent experiments. *Statistically significant difference compared to the negative control sample (C(-)) (Mann–Whitney *U*-test), *P < 0.05.

Determination of Metalloproteinase 2 and 9 Levels

This study used the same concentrations of *P. ficariae* and *W. bliti* extracts to induce proinflammatory cytokines. The level of metalloproteinases was determined after 24 hours of incubation of the cells with the selected concentrations of the fungal extracts.

Both the *P. ficariae* and *W. bliti* extracts slightly increased MMP-2 and MMP-9 production by the A549 and BEAS-2B cells only at their lower concentrations; however, the differences were not statistically significant (Figure 9A and B).

Evaluation of Epithelial Cell Integrity

To detect the presence of intercellular junction proteins E-cadherin and occludin, the same concentrations of the *P. ficariae* and *W. bliti* extracts were used to determine cytokine production.

In the lung alveolar epithelial cells, the extract at a concentration of 83 µg protein/mL seemed to be much more effective in reducing the presence of E-cadherin and occludin, as confirmed by the lower green fluorescence intensity of both cell integrity markers than in the A549 cell monolayers stimulated with 50 µg protein/mL of the *P. ficariae* extract (Figure 10A). The E-cadherin fluorescence intensity in the BEAS-2B cells was significantly lower in cells exposed to the

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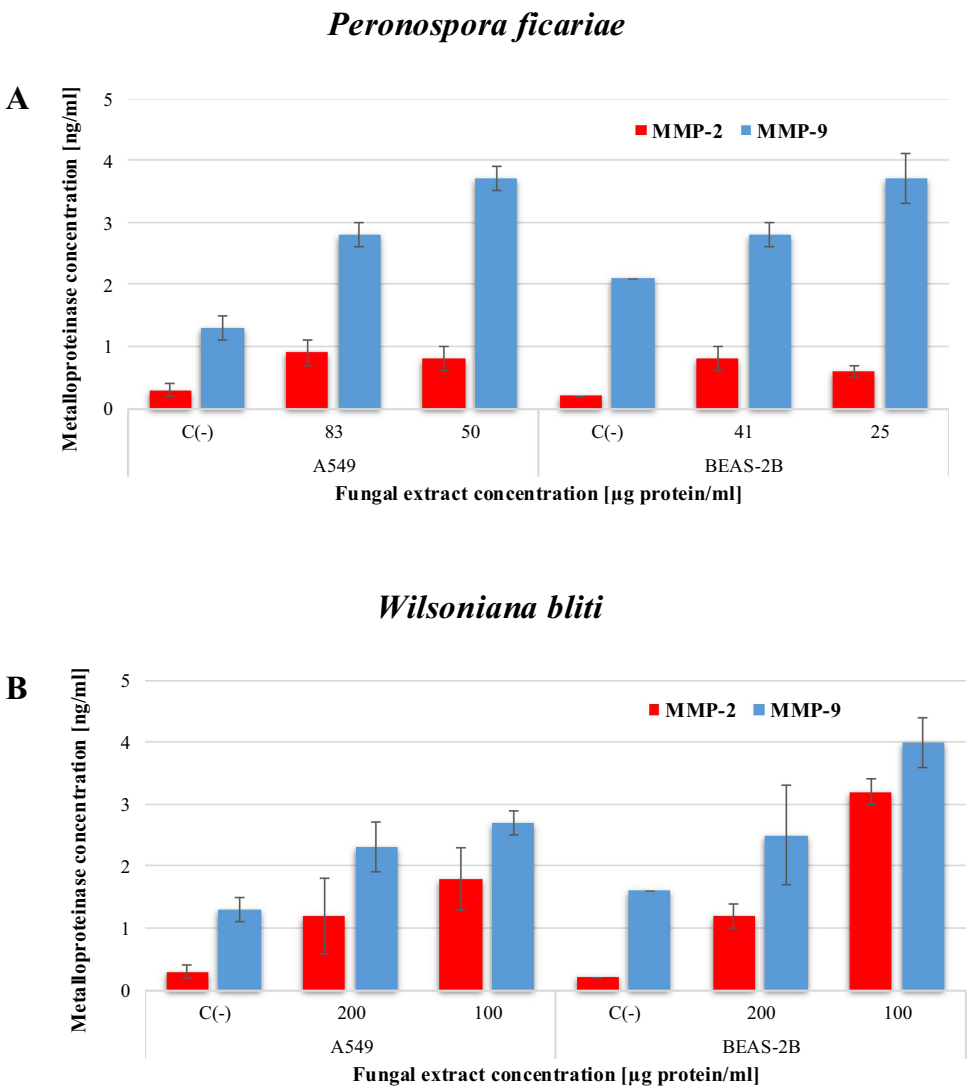

Figure 9 Assessment of MMP-2 and MMP-9 production by A549 and BEAS-2B cells after 24-hour incubation with *P. ficariae* (**A**) and *W. bliti* (**B**) fungal extracts. The results are mean ± SD values from three independent experiments. C(-) – negative control sample. No statistically significant differences were found compared to the control (Mann–Whitney *U*-test).

P. ficariae extract at a concentration of 41 µg protein/mL than 25 µg protein/mL, suggesting a significant reduction in the presence of this protein and, therefore, weaker cell–cell adhesion. In contrast to E-cadherin, the presence of occludin was significantly affected by the 24-hour incubation of the bronchial epithelial cells with the lower concentration of the fungal extract (Figure 10B).

The E-cadherin and occludin staining in the A549 cells exposed to the *W. bliti* extract revealed more discrete and limited fluorescence of these proteins than in the control cells. Its intensity decreased in direct proportion to the increase in the concentration of the *W. bliti* extract (Figure 10A). Similar to the A549 cells, differences were observed in the bronchial epithelial cell monolayers – a trend towards lower levels of cellular fluorescence intensity of E-cadherin and occludin was observed at the *W. bliti* extract concentration of 200 µg protein/mL (Figure 10B).

Discussion

Allergies are increasingly being recognized as a 21st-century epidemic, with their occurrence steadily rising. The World Allergy Organization 2013 White Book on Allergy reports that allergy prevalence varies from 10% to 40% depending on the country.

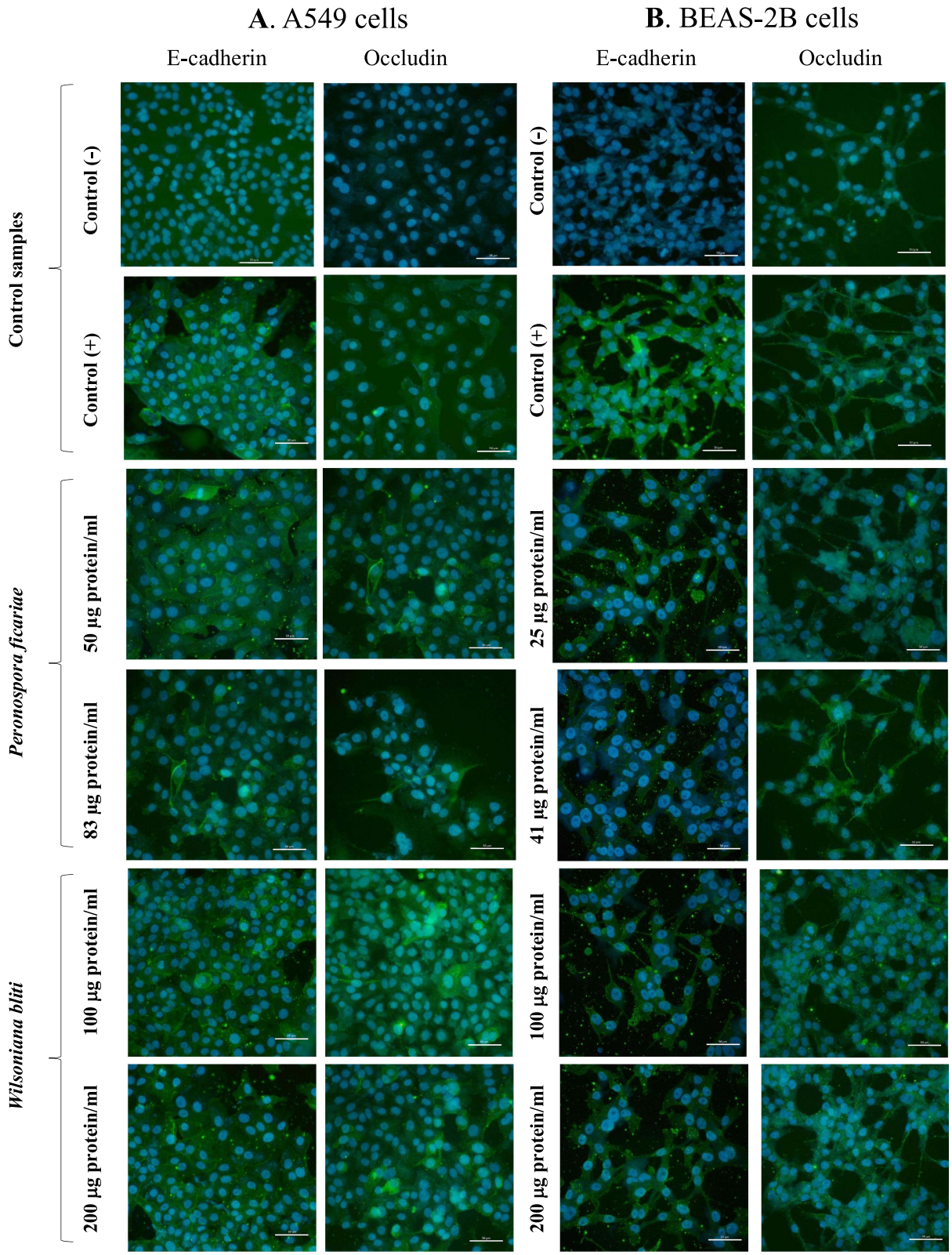

Figure 10 Effect of *P. ficariae* and *W. bliti* extracts on the presence of E-cadherin and occludin in A549 (**A**) and BEAS-2B (**B**) cells (representative photos from three independent replicates). Control (-) - fungal extract-untreated cells incubated with secondary antibody only; Control (+) - fungal extract-untreated cells incubated with primary and secondary antibody. Cell nuclei stained with DAPI (blue). Bars: 50 µm.

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Around 200–250 million individuals experience food allergies, one-tenth of the population reacts adversely to medications, and the estimated number of people affected by asthma and allergic rhinitis is 300 million and 400 million, respectively.[30]

Since the 12th century, fungal spores have been considered major inhalant allergens.[31] Although the precise prevalence of fungal allergies is not clearly defined, it is estimated to affect between 3% and 10% of the population, varying with the region's climate.[32] The most thoroughly documented allergenic characteristics are found in common fungi from the genera *Cladosporium, Penicillium, Aspergillus*, and *Alternaria*.[33] *C. herbarum* and *A. alternata* rank as the third most prevalent inhalant allergens, following house dust and grass pollen, highlighting the importance of assessing the potential risks of atmospheric fungal spores.[34] These spores trigger IgE-mediated type I hypersensitivity reactions, such as allergic rhinitis and asthma. Higher concentrations of these spores in the air are linked to increased asthma-related hospital admissions and fatalities as well as a greater likelihood of developing rhinitis.[32,35] Fungi can also cause other respiratory conditions, including bronchopulmonary mycoses, allergic sinusitis, and hypersensitivity pneumonitis.[36,37]

For the first time, we present two phytopathogenic fungus-like organisms belonging to the orders Peronosporales (*Peronospora ficariae*) and Albuginales (*Wilsoniana bliti*), which parasitize common plants *Ficaria verna* and *Amaranthus retroflexus*, respectively. In an in vitro study utilizing an upper respiratory tract cellular model, we demonstrated their proinflammatory and, therefore, allergenic potential. Additionally, to support the microscopic identification of the studied fungal species, we describe their molecular identification.

Currently, in addition to the analysis based on the sequence of the nrDNA regions LSU, ITS1, and ITS2, other barcodes, such as genes encoding cytochrome oxidase subunits 1 and 2 (*cox1* and *cox2*), calmodulin (CaM), or beta-tubulin, are increasingly being considered in determining the taxonomic position of fungi. Combining the results of LSU or ITS sequence analysis with other molecular markers is often necessary to establish the species position, facilitating better identification of the organism at the species level and beyond.[29,38]

The phylogenetic studies allowed us to determine the taxonomic position of the two fungal isolates. Based on the comparative analysis of the combined sequences of the ITS and LSU regions as well as the cox1 and cox2 gene sequences, the isolate from *Ficaria verna* was identified as the species *P. ficariae*. The comparative sequence analysis of the ITS and LSU regions, along with genes encoding cytochrome oxidase subunits 1 and 2 (*cox1* and *cox2*) from the second strain, which was obtained from *Amaranthus retroflexus*, indicated that the isolate belongs to the species *W. bliti*.

An important element in the pathogenesis and progression of respiratory allergic disease is the penetration of spores into the body through inhalation, facilitated by their small size.[33] Fungal spores are orders of magnitude smaller than most moss and fern spores and are comparable in size to some plant pollens.[39] They generally have a diameter ranging from 2 µm to 50 µm, with most allergenic spores having a respirable diameter between 3 and 10 µm.[33] Fungi of the genus *Aspergillus* produce large amounts of small airborne conidia measuring 2–5 µm in diameter, with approximately 100–1000 per day inhaled by humans. Due to their small size, they can reach the pulmonary alveoli and induce the development of hypersensitivity reactions, such as asthma or allergic bronchopulmonary aspergillosis (ABPA).[40] Although the size of *P. ficariae* and *W. bliti* conidia and conidiophores exceeds 10 µm, their penetration into the bronchi and alveoli in humans is also probable since even larger fungal hyphae are inhalable.[41]

Like other allergic conditions, asthma is rooted in inflammatory responses marked by an abnormal immune reaction that impacts epithelial cells, fibroblasts, vascular cells, and airway smooth muscle cells. These cells and immune cells become significant sources of inflammatory mediators. Research has highlighted the role of cytokines derived from epithelial cells in encouraging Th2 immune responses.[42] Numerous studies have pinpointed the critical involvement of airway epithelial-derived cytokines in the development of asthma, such as the proinflammatory IL-1β and IL-6, GM-CSF (which aids in the recruitment of granulocytes and monocytes), TNF-α (which maintains epithelial integrity), and TGFβ (which is involved in airway remodeling).[43,44]

Our laboratory studies confirmed that epithelial airway cells generated proinflammatory cytokines when exposed to extracts from *P. ficariae* and *W. bliti*. To assess the proinflammatory and allergenic properties of *P. ficariae* and *W. bliti* regarding airway cells, we used two cell models commonly employed in such research: human normal bronchial epithelial cells (BEAS-2B[22] and human lung carcinoma epithelial cells (A549).[22–24] While the extract of *P. ficariae* induced IL-1β and GM-CSF in the bronchial epithelial cells (BEAS-2B line) and only GM-CSF in the human lung

carcinoma epithelial cells (A549 line), *W. bliti* induced these cytokines exclusively in the BEAS-2B cell line, particularly at the lower extract concentrations.

In similar experiments conducted with *Erysiphe convolvuli* and *E. palczewskii*, which are microfungi parasitizing common plants *Convolvulus arvensis* and *Caragana arborescens*, we observed their ability to induce proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in bronchial (BEAS-2B line) and alveolar human epithelial cells (A549 line).[45] Additionally, in our earlier studies, we presented that *Tranzschelia pruni-spinosae*, which parasitizes *Prunus domestica*, and *Phragmidium rubi-idaei*, which infests *Rubus idaeus*, were also able to induce proinflammatory cytokines in the same model of the upper and lower respiratory tract.[46]

In contrast, other researchers found that, in the BEAS-2B cell line, hyphal fragments from *A. fumigatus* and *P. chrysogenum* led to an increase in IL-1α and TNF-α expression but did not trigger their release;[47] meanwhile, when exposed to conidia or mycelial fragments of *A. fumigatus*, A549 cells showed elevated production of TNF-α and GM-CSF.[41,42] This suggests that the inflammatory response may vary depending on the fungal species and the type of cells involved.

Airway epithelial cells are part of the primary defense mechanism against inflammatory stimuli and antigens in the airway and lungs.[48] In asthma, epithelial disruption is a notable feature facilitating easier passage of inhaled substances into the airway wall, where they can interact with immune and inflammatory cells.[49] The respiratory tract frequently encounters infectious agents like viruses and bacteria as well as plant and fungal spores in the atmosphere. These elements can compromise the integrity of the respiratory barrier, playing a role in the onset and progression of diseases. Tight junctions (TJ) in the airway epithelium, which include E-cadherin and occludin proteins, are crucial for maintaining the airway's continuous mechanical barrier.[50] The indirect effects of various allergens (mites, pollen, cat, dog, fungal) can lead to reduced levels of these proteins in asthma due to their protease activity[42,51] or capacity to generate harmful reactive oxygen species.[52,53]

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that play several essential roles in physiological processes, such as remodeling the extracellular matrix, aiding cell migration, and cleaving cytokines. Typically, MMPs are not found in normal, healthy tissues but in inflamed tissues or those undergoing repair and remodeling. Pulmonary epithelial cells might also be a notable source of MMPs, as they express MMP-1, -2, -7, and -9, although their expression profile may vary depending on the stimulus.[54–56] Some evidence suggests that MMPs are involved in the pathophysiology of fungal infections. As reported by Saadat et al, an extract from *A. fumigatus* inhibited the production of MMPs in fibrosarcoma cell lines, as demonstrated by gelatin zymography.[57] In contrast to those studies, the *T. pruni-spinosae* and *P. rubi-idaei* extracts used in our previous experiments only slightly increased MMP-9 levels in both A549 and BEAS-2B cells, as indicated by ELISA tests.[46] Also, in the present study with *P. ficariae* and *W. bliti* extracts, we did not show enhanced MMP-2 and MMP-9 production in the A549 and BEAS-2B cell lines.

Numerous studies have indicated that inhaling various allergens, eg fungi, can lead to the production of ROS.[58,59] For instance, the inflammatory response associated with *Aspergillus* protease triggers mitochondrial ROS generation in A549 cells. Likewise, *A. fumigatus* conidia have been found to produce oxygen species in BEAS-2B cells.[60] We also indicated earlier that *E. palczewskii, E. convolvuli*, and *T. pruni-spinosae* induced ROS production in both BEAS-2B and A549 cells used as a model of the upper and lower respiratory tract, respectively.[45,46] In the present experiments, only the *P. ficariae* extract was able to induce ROS generation in both model cell lines (A549 and BEAS-2B).

Exposure to inflammatory cytokines is another factor that can disrupt the epithelial barrier. Petecchia et al found that contact with TNF-α, IL-4, and IFN-γ led to a significant decrease in occludin levels in airway cells.[61] A similar role of TNF-α and IFN-γ in increasing airway cell permeability by disrupting claudin-mediated junctions in in vitro studies was described by Capaldo et al.[62] In human bronchial epithelial cells (HBEC), TNF-α resulted in the loss of occludin and claudins from tight junctions, along with the redistribution of E-cadherin.[63] A recent study conducted by Sztandera-Tymoczek reported that *E. palczewskii* and *E. convolvuli* triggered a notable release of IL-1β and TNF-α, which may be linked to the diminished visibility of E-cadherin and occludin in A549 and BEAS-2B cells.[45] In our study, *P. ficariae* induced IL-1β and ROS production generally more efficiently than the *W. bliti* extract, which was possibly related to the reduced visibility of the intercellular junction proteins E-cadherin and occludin in both cell lines.

In our in vitro experiments, in contrast to *W. bliti*, which did not present proinflammatory activity, the lower concentrations of the *P. ficariae* extract caused a more substantial inflammatory effect in most cases. A comparable

pattern was identified in *P. chrysogenum*, a species with well-documented allergenic characteristics. Oya et al reported that when bronchial epithelial cells (BEAS-2B) were subjected to different concentrations of *P. chrysogenum* hyphae, the release of IL-1β, TNF-α, IL-6, and IL-1α was slightly more pronounced at a concentration of 50 μg/mL, compared to 100 μg/mL.[47] Kauffman et al found a similar pattern in the A549 cell line, where IL-6 and IL-8 cytokine levels were elevated in response to *A. alternata* fungal extract at concentrations of 50, 100, or 200 μg/mL, compared to the highest concentration tested, which was 400 μg/mL. Additionally, cytokine production was most pronounced at the lowest concentration of 50 μg/mL of *A. fumigatus* fungal extract.[64]

While fungal spores are considered major inhalant allergens,[31] the precise rate of fungal allergies remains unclear. Additionally, standard skin or blood tests do not always pinpoint the allergen responsible for an existing allergy, indicating that there may be undiscovered allergens. Our study is the first to suggest that the fungus-like organism *P. ficariae*, which parasitizes common plants, such as *Ficaria verna*, may be a new source of human allergies, thus broadening the known range of clinically used standards of fungal allergens. Additionally, we showed that *W. bliti* infesting *Amaranthus retroflexus* did not present significant proinflammatory activities in in vitro studies.

Conclusions

In our current in vitro experiments, we demonstrated that the two studied fungal extracts, mainly *P. ficaria*, have the potential to jointly compromise the epithelial barrier in both the upper and lower respiratory tracts. This occurs through the induction of proinflammatory cytokines and the generation of reactive oxygen species, also possibly through low levels of metalloproteinases. While none of these factors were exceptionally elevated, their combined effect was able to reduce the levels of tight junction proteins, such as E-cadherin and occludin, within epithelial cells, suggesting a potential environment for allergic reaction development. Consequently, it is plausible to suggest that growers or gardeners might experience allergic reactions when there is a significant infection of *Ficaria verna* by *P. ficariae* or *Amaranthus retroflexus* by *W. bliti*. Our research contributes to understanding new potential respiratory allergens, which still need confirmation through in vivo studies.

Limitations of the Study

However, our study has certain limitations. One is the use of crude microfungal extracts, which must be standardized to pinpoint the most active components, such as proteins, lipids, or their complexes. Another limitation is the necessity to validate our findings using an animal model. Future research should address these limitations to thoroughly elucidate the mechanisms and implications of new fungal allergens in human allergic responses.

Data Sharing Statement

The article contains all the necessary data pertaining to the study. The original findings presented in the research are included in the article. For additional inquiries, please contact the corresponding author.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas, took part in drafting, revising, or critically reviewing the article, gave final approval of the version to be published, have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted without any commercial or financial relationship that could be construed as a potential conflict of interest.

References

1. Pawankar R. Allergic diseases and asthma: a global public health concern and a call to action. *World Allergy Organ J*. 2014;7(1):12. doi:10.1186/1939-4551-7-12
2. Gutowska-Slesik J, Samolinski B, Krzych-Falta E. The increase in allergic conditions based on a review of literature. *Postepy Dermatol Alergol*. 2023;40(1):1–7. doi:10.5114/ada.2022.119009
3. (EAACI) TEAoAaCI. 2024. Available from: https://eaaci.org/wp-content/uploads/2024/02/EAACI_Advocacy_Manifesto.pdf. Accessed July 30, 2025.
4. EFA Book on Respiratory Allergies Raise Awareness RtB. Available from: https://www.efanet.org/images/documents/EFABookonRespiratoryAllergiesFINAL.pdf. Accessed June 10, 2025.
5. Crameri R, Garbani M, Rhyner C, Huitema C. Fungi: the neglected allergenic sources. *Allergy*. 2014;69(2):176–185. doi:10.1111/all.12325
6. Zukiewicz-Sobczak WA. The role of fungi in allergic diseases. *Postepy Dermatol Alergol*. 2013;30(1):42–45. doi:10.5114/pdia.2013.33377
7. Horner WE, Helbling A, Salvaggio JE, Lehrer SB. Fungal allergens. *Clin Microbiol Rev*. 1995;8(2):161–179. doi:10.1128/CMR.8.2.161
8. Cecchi L, D'Amato G, Ayres JG, et al. Projections of the effects of climate change on allergic asthma: the contribution of aerobiology. *Allergy*. 2010;65(9):1073–1081. doi:10.1111/j.1398-9995.2010.02423.x
9. Sztandera-Tymoczek M, Szuster-Ciesielska A. Fungal Aeroallergens-The Impact of Climate Change. *J Fungi*. 2023;9(5). doi:10.3390/jof9050544
10. Fukutomi Y, Taniguchi M. Sensitization to fungal allergens: resolved and unresolved issues. *Allergol Int*. 2015;64(4):321–331. doi:10.1016/j.alit.2015.05.007
11. Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *J Bacteriol*. 1990;172(8):4238–4246. doi:10.1128/jb.172.8.4238-4246.1990
12. Pfunder M, Roy BA. Pollinator-mediated interactions between a pathogenic fungus, Uromyces pisi (Pucciniaceae), and its host plant, Euphorbia cyparissias (Euphorbiaceae). *Am J Bot*. 2000;87(1):48–55. doi:10.2307/2656684
13. Robideau GP, De Cock AW, Coffey MD, et al. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Mol Ecol Resour*. 2011;11(6):1002–1011. doi:10.1111/j.1755-0998.2011.03041.x
14. Hudspeth DSS, Nadler SA, Hudspeth MES. A COX2 molecular phylogeny of the Peronosporomycetes. *Mycologia*. 2000;92(4):674–684. doi:10.1080/00275514.2000.12061208
15. Aime MC. Toward resolving family-level relationships in rust fungi (Uredinales). *Mycoscience*. 2006;47:112–122. doi:10.1007/s10267-006-0281-0
16. Thompson JDHDG, Gibson TJ, Gibson TJ. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994;22:4673–4680. doi:10.1093/nar/22.22.4673
17. Nicholas KBNHBJ. *GeneDoc*. Pittsburgh:Pittsburgh Supercomputing Center; 1997.
18. Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol*. 2021;38(7):3022–3027. doi:10.1093/molbev/msab120
19. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods*. 2012;9(8):772. doi:10.1038/nmeth.2109
20. Page RD. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci*. 1996;12:357–358. doi:10.1093/bioinformatics/12.4.357
21. Menezes EA, Gambale W, Macedo MS, Abdalla DS, Paula CR, Croce J. Biochemical, antigenic and allergenic characterization of crude extracts of Drechslera (Helminthosporium) monoceras. *Mycopathologia*. 1995;131(2):75–81. doi:10.1007/BF01102882
22. Verstraelen S, Bloemen K, Nelissen I, Witters H, Schoeters G, Van Den Heuvel R. Cell types involved in allergic asthma and their use in in vitro models to assess respiratory sensitization. *Toxicol In Vitro*. 2008;22(6):1419–1431. doi:10.1016/j.tiv.2008.05.008
23. Blume C, Foerster S, Gilles S, et al. Human epithelial cells of the respiratory tract and the skin differentially internalize grass pollen allergens. *J Invest Dermatol*. 2009;129(8):1935–1944. doi:10.1038/jid.2008.459
24. Crossen AJ, Ward RA, Reedy JL, et al. Human airway epithelium responses to invasive fungal infections: a critical partner in innate immunity. *J Fungi*. 2022;9(1). doi:10.3390/jof9010040
25. Zhao F, Klimecki WT. Culture conditions profoundly impact phenotype in BEAS-2B, a human pulmonary epithelial model. *J Appl Toxicol*. 2015;35(8):945–951. doi:10.1002/jat.3094
26. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1–2):55–63. doi:10.1016/0022-1759(83)90303-4
27. Wojciechowska A, Bregier Jarzebowska R, Komarnicka UK, et al. Isothiocyanate l-argininato copper(II) complexes - Solution structure, DNA interaction, anticancer and antimicrobial activity. *Chem Biol Interact*. 2021;348:109636. doi:10.1016/j.cbi.2021.109636
28. Polska KJF. Grzyby (Mycota) 4. Glonowce (Phycomycetes), wroślikowe (Peronosporales). *PWN Warszawa-Kraków*. 1970.
29. Choi YJ, Beakes G, Glockling S, et al. Towards a universal barcode of oomycetes–a comparison of the cox1 and cox2 loci. *Mol Ecol Resour*. 2015;15(6):1275–1288. doi:10.1111/1755-0998.12398
30. Available from: https://www.worldallergy.org/wao-white-book-on-allergy. Accessed July 30, 2025.
31. Lin WR, Chen YH, Lee MF, et al. Does spore count matter in fungal allergy?: the role of allergenic fungal species. *Allergy Asthma Immunol Res*. 2016;8(5):404–411. doi:10.4168/aair.2016.8.5.404
32. Twaroch TE, Curin M, Valenta R, Swoboda I. Mold allergens in respiratory allergy: from structure to therapy. *Allergy Asthma Immunol Res*. 2015;7(3):205–220. doi:10.4168/aair.2015.7.3.205
33. Patel TY, Buttner M, Rivas D, Cross C, Bazylinski DA, Seggev J. Variation in airborne fungal spore concentrations among five monitoring locations in a desert urban environment. *Environ Monit Assess*. 2018;190(11):634. doi:10.1007/s10661-018-7008-5
34. Abel-Fernandez E, Martinez MJ, Galan T, Pineda F. Going over fungal allergy: Alternaria alternata and Its Allergens. *J Fungi*. 2023;9(5). doi:10.3390/jof9050582
35. Jaakkola MS, Quansah R, Hugg TT, Heikkinen SA, Jaakkola JJ. Association of indoor dampness and molds with rhinitis risk: a systematic review and meta-analysis. *J Allergy Clin Immunol*. 2013;132(5):1099–1110e18. doi:10.1016/j.jaci.2013.07.028
36. Simon-Nobbe B, Denk U, Poll V, Rid R, Breitenbach M. The spectrum of fungal allergy. *Int Arch Allergy Immunol*. 2008;145(1):58–86. doi:10.1159/000107578

37. Baxi SN, Portnoy JM, Larenas-Linnemann D, Phipatanakul W, Environmental Allergens W. Exposure and health effects of fungi on humans. *J Allergy Clin Immunol Pract*. 2016;4(3):396–404. doi:10.1016/j.jaip.2016.01.008
38. Ezeonuegbu BA, Abdullahi MD, Whong CMZ, et al. Characterization and phylogeny of fungi isolated from industrial wastewater using multiple genes. *Sci Rep*. 2022;12(1):2094. doi:10.1038/s41598-022-05820-9
39. Golan JJ, Pringle A. Long-distance dispersal of fungi. *Microbiol Spectr*. 2017;5(4). doi:10.1128/microbiolspec.FUNK-0047-2016
40. Palmieri F, Koutsokera A, Bernasconi E, Junier P, von Garnier C, Ubags N. Recent advances in fungal infections: from lung ecology to therapeutic strategies with a focus on Aspergillus spp. *Front Med*. 2022;9:832510. doi:10.3389/fmed.2022.832510
41. Luo Y, Liu F, Deng L. Innate and adaptive immune responses induced by aspergillus fumigatus conidia and hyphae. *Curr Microbiol*. 2023;80. doi:10.1007/s00284-022-03102-1.
42. Georas SN, Rezaee F. Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. *J Allergy Clin Immunol*. 2014;134(3):509–520. doi:10.1016/j.jaci.2014.05.049
43. Gandhi VD, Vliagoftis H. Airway epithelium interactions with aeroallergens: role of secreted cytokines and chemokines in innate immunity. *Front Immunol*. 2015;6:147. doi:10.3389/fimmu.2015.00147
44. Lambrecht BN, Hammad H, Fahy JV. The Cytokines of Asthma. *Immunity*. 2019;50(4):975–991. doi:10.1016/j.immuni.2019.03.018
45. Sztandera-Tymoczek M, Wdowiak-Wrobel S, Swiderska U, Palusinska-Szysz M, Szuster-Ciesielska A. Potential proallergenic activity of phytopathogenic erysiphe palczewskii and erysiphe convolvuli in in vitro studies. *J Inflamm Res*. 2023;16:5039–5060. doi:10.2147/JIR.S425383
46. Sztandera-Tymoczek M, Wdowiak-Wrobel S, Swiderska U, Palusinska-Szysz M, Szuster-Ciesielska A. The Potential Proallergenic Activity of Tranzschelia pruni-spinosae and Phragmidium rubi-idaei in vitro Studies. *J Inflamm Res*. 2025;18:1107–1125. doi:10.2147/JIR.S497219
47. Oya E, Becher R, Ekeren L, Afanou AKJ, Ovrevik J, Holme JA. Pro-inflammatory responses in human bronchial epithelial cells induced by spores and hyphal fragments of common damp indoor molds. *Int J Environ Res Public Health*. 2019;16(6):1085. doi:10.3390/ijerph16061085
48. Yuksel H, Ocalan M, Yilmaz O. E-cadherin: an important functional molecule at respiratory barrier between defence and dysfunction. *Front Physiol*. 2021;12:720227. doi:10.3389/fphys.2021.720227
49. Wang Y, Bai C, Li K, Adler KB, Wang X. Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respir Med*. 2008;102(7):949–955. doi:10.1016/j.rmed.2008.01.017
50. Heijink IH, Kuchibhotla VNS, Roffel MP, et al. Epithelial cell dysfunction, a major driver of asthma development. *Allergy*. 2020;75(8):1902–1917. doi:10.1111/all.14421
51. Matsumura Y. Role of allergen source-derived proteases in sensitization via airway epithelial cells. *J Allergy*. 2012;2012:903659. doi:10.1155/2012/903659
52. Yamaya M, Sekizawa K, Masuda T, Morikawa M, Sawai T, Sasaki H. Oxidants affect permeability and repair of the cultured human tracheal epithelium. *Am J Physiol*. 1995;268(2 Pt 1):L284–93. doi:10.1152/ajplung.1995.268.2.L284
53. Zhang Q, Lin JL, Thomas PS. Reactive Oxygen Species and Obstructive Lung Disease. In: Laher Ie, editor. *Systems Biology of Free Radicals and Antioxidants*. Springer; 2014:1643–1670.
54. Vermeer PD, Denker J, Estin M, et al. MMP9 modulates tight junction integrity and cell viability in human airway epithelia. *Am J Physiol Lung Cell Mol Physiol*. 2009;296(5):L751–62. doi:10.1152/ajplung.90578.2008
55. Yao PM, Buhler JM, d'Ortho MP, et al. Expression of matrix metalloproteinase gelatinases A and B by cultured epithelial cells from human bronchial explants. *J Biol Chem*. 1996;271(26):15580–15589. doi:10.1074/jbc.271.26.15580
56. Elkington PT, Friedland JS. Matrix metalloproteinases in destructive pulmonary pathology. *Thorax*. 2006;61(3):259–266. doi:10.1136/thx.2005.051979
57. Saadat F, Zomorodian K, Pezeshki M, Rezaie S, Khorramizadeh MR. Inhibitory effect of Aspergillus fumigatus extract on matrix metalloproteinases expression. *Mycopathologia*. 2004;158(1):33–37. doi:10.1023/b:myco.0000038429.46699.ac
58. Warris A, Ballou ER. Oxidative responses and fungal infection biology. *Semin Cell Dev Biol*. 2019;89:34–46. doi:10.1016/j.semcdb.2018.03.004
59. Wang X, Che MZ, Khalil HB, et al. The role of reactive oxygen species in the virulence of wheat leaf rust fungus Puccinia triticina. *Environ Microbiol*. 2020;22(7):2956–2967. doi:10.1111/1462-2920.15063
60. Kastelberg B, Ayubi T, Tubau-Juni N, et al. Nlrx1-regulated defense and metabolic responses to aspergillus fumigatus are morphotype and cell type specific. *Front Immunol*. 2021;12:749504. doi:10.3389/fimmu.2021.749504
61. Petecchia L, Sabatini F, Usai C, Caci E, Varesio L, Rossi GA. Cytokines induce tight junction disassembly in airway cells via an EGFR-dependent MAPK/ERK1/2-pathway. *Lab Invest*. 2012;92(8):1140–1148. doi:10.1038/labinvest.2012.67
62. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. *Biochim Biophys Acta*. 2009;1788(4):864–871. doi:10.1016/j.bbamem.2008.08.027
63. Hardyman MA, Wilkinson E, Martin E, et al. TNF-alpha-mediated bronchial barrier disruption and regulation by src-family kinase activation. *J Allergy Clin Immunol*. 2013;132(3):665–675e8. doi:10.1016/j.jaci.2013.03.005
64. Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J Allergy Clin Immunol*. 2000;105(6 Pt 1):1185–1193. doi:10.1067/mai.2000.106210