*Journal of Food and Nutrition Research, 2025, Vol. 13, No. 4, 181-189*
Available online at http://pubs.sciepub.com/jfnr/13/4/2
Published by Science and Education Publishing
DOI:10.12691/jfnr-13-4-2

# Honey Protects Against PM2.5-induced Damage Through Its Anti-inflammatory Effects in Human Lung Bronchial Epithelial Cells

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*Received March 16, 2025; Revised April 18, 2025; Accepted April 25, 2025*

**Abstract** Particulate matter with an aerodynamic diameter of ≤ 2.5 μm (PM2.5) can cause pulmonary injury and has emerged as a public health concern. Honey, a nutritional health product used in traditional medicine, has been shown to be effective for clinical applications ranging from wound healing to cancer treatment. Honey contains phenolics and flavonoids that have been extensively studied for their clinical activity in inflammation-mediated chronic diseases, as well as their ability to reduce inflammation in bronchial tubes (airways within the lungs). This study aimed to evaluate the protective effects of different honey samples against PM2.5-induced damage in the human lung epithelial cell line BEAS-2B. The results showed that honey pre-treatment markedly inhibited PM2.5-induced risk factors for chronic obstructive pulmonary disease through anti-inflammatory activity and protection of the epithelial barrier. Honey pre-treatment restored the expression of zonula occludens (ZO)-1, ZO-2, and alpha-1 antitrypsin in BEAS-2B cells after PM 2.5 treatment and downregulated the expression of the inflammatory factor interleukin (IL)-8 by more than 70%. These findings demonstrate the protective effects of honey against PM2.5-induced epithelial barrier damage and inflammation in BEAS-2B lung epithelial cells. Therefore, honey may be a promising compound for preventing PM2.5-triggered cell damage.

***Keywords:*** *PM2.5, honey, lung epithelial cell, anti-inflammatory, chronic obstructive pulmonary disease, ZO-1/ZO-2, AAT*

**Cite This Article:** Hung-Chun Lu, Hui-Qi Liu, Ming-Cheng Wu, and Chi-Chung Peng, "Honey Protects Against PM2.5-induced Damage Through Its Anti-inflammatory Effects in Human Lung Bronchial Epithelial Cells." *Journal of Food and Nutrition Research*, vol. 13, no. 4 (2025): 181-189. doi: 10.12691/jfnr-13-4-2.

## 1. Introduction

Fine particulate matter with diameter ≤ 2.5 μm (PM2.5) is a major air pollutant that primarily consists of metals, inorganic molecules, polycyclic aromatic hydrocarbons, and other particles [1]. Due to its small size PM2.5 can penetrate the human lung and even reach the alveolar region. Numerous epidemiologic studies have indicated a positive association between PM2.5 pollution and pulmonary, cardiovascular, and immune-mediated diseases, including cancer [2]. PM2.5 pollution is ubiquitous, especially in urban environments. Therefore, development of measures to prevent the health hazards caused by these particles is essential.

Exposure to PM2.5 has been linked to acute and chronic respiratory diseases in numerous epidemiological studies. For toxicity assessments, in vitro and in vivo models based on the lungs are frequently used because of the importance of the lungs as the main respiratory organ and the main target of ambient air contaminants. Previous studies have suggested that the oxidative stress, inflammation, and epithelial injury caused by PM2.5 contribute to lung disease pathogenesis [3,4].

Airway epithelial cells act as a natural barrier to prevent inhaled xenobiotics from reaching tissues [5]. However, excessive ROS accumulation decreases the levels of tight-junction proteins such as zonula occludens (ZO) and occludin, resulting in further disruption of the lung epithelial barrier [6]. According to Lucas et al., [7] a diseased epithelial barrier may serve as a potential marker for the early identification of pulmonary disease, and exposure to PM2.5 may increase the permeability of the epithelial barrier [8]. According to Barnes et al., [9] a reduction in the protease-antiprotease ratio indicates pathogenesis, and proteases degrade lung connective tissues to cause chronic obstructive pulmonary disease (COPD) [10]. Alpha-1 antitrypsin (AAT; assay serpin peptidase inhibitor, clade A, member 1) is an elastase inhibitor that protects the alveoli and is primarily produced by bronchial epithelial cells, hepatocytes, and macrophages [11,12]. AAT oxidation due to excess ROS reduces its ability to protect barrier integrity and

converts it into an inducer of pro-inflammatory cytokines [13]. COPD may accelerate the progression of these responses [13,14].

Honey is a naturally sweet substance produced by honeybees from blossom nectar and plant exudates, depending on the type of nectar [15]. Honey shows a variety of sensory and physicochemical characteristics depending on the climate, environmental conditions, plant origin, and honeybee species. Honey has also been reported to show several benefits, including antioxidant [15,16], antiproliferative [17], antibacterial [18], and anti-inflammatory effects [15,19]. The biological and medical properties of honey contribute to its ability to prevent certain acute and chronic diseases, including bronchitis, respiratory distress, and diabetes [20].

Honey is mainly produced by honeybees and stingless bees, of which honeybees include Apis mellifera, Apis cerana, and the wild bee Apis dorsata [21]. Both types of honey contain distinct flavonoids and phenolic compounds, which have recently been reported to show biological and clinical effects on inflammation-mediated chronic diseases [22,23]. Therefore, in this study, the protective effects of various honey samples against PM2.5-induced damage were studied in human lung epithelial BEAS-2B cells.

## 2. Materials and Methods

### 2.1. Honey Samples

Nine honey samples were collected from different bee species: three from *A. mellifera*, three from stingless honeybees (*Heterotrigona itama*, *Tetrigona binghami*, and *Tetragonula laeviceps*), two from *A. cernana*, and one from *A. dorsata*. The three *A. mellifera* honey samples collected from different floral species (lychee, longana, and multifloral), and the two *A. cernana* honey samples obtained from different locations (Taipei [Cerana1]) and Chiayi [Cerana2]) were provided by YONG SHYANG HONEY ENTERPRISE Co., Ltd. (Changhua, Taiwan). The three stingless bee honey samples were obtained from Kuok Kiang Tiong at the bee farm of BEE EXC SCI TECK SDN. BHD in Sibu, Sarawak, Malaysia during the nectar flow period in September and October 2023. The *A. dorsata* honey sample from Sumatra, Indonesia, was purchased from a market.

### 2.2. Physicochemical Analysis of the Honey Samples

Water content was determined using an infrared (drying) moisture meter (Kett FD-720, Tokyo, Japan). The fructose, glucose, trehalulose, and sucrose contents of the honey samples were analysed by high-pressure liquid chromatography (Milford, Massachusetts, USA) with a refractive index detector (Waters TaperSlit, USA) using modified versions of previously reported methods [24,25]. A 5% (w/v) solution of honey in distilled water was filtered through a 0.45-µm filter paper, and 10 µL of the filtered sample was loaded onto a Supercosil LC-NH2 column (4.6 × 250 mm, 5 µm, Merck, Darmstadt, Germany) with 75% acetonitrile as the mobile phase at a flow rate of 1.0 mL/ min and an oven temperature of 40°C. Sugar peaks were identified by comparing the retention times obtained from the reference standards. The sugar content of the honey samples was calculated using the standard curve for each reference sugar.

The protein content of the honey samples was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. The diastase activity was determined using a honey diastase test kit (Phadebas, Cambridge, MA, USA) and a photometric method, wherein the absorbance of the analyzed solutions at 620 nm was directly proportional to the diastase activity of the honey samples. The total acidity was determined by titration of honey (5.0 g) dissolved in 100 mL of distilled water with 0.1 N NaOH to pH 8.3, with the results expressed as mEq/kg [26]. The pH of a solution of 5 g of honey in 100 mL of ultrapure water was measured using a pH meter (Jenco, Taipei, Taiwan).

Diastase activity in the honey samples was measured using Phadebas amylase test tablets purchased from Magle (Lund, Sweden) according to the International Honey Commission [27]. Diastase activity was referred to as diastase number in the Schade scale, which corresponded to the Gothe scale number, or hydrolyzed starch (g)/100 g of honey/hour at 40°C. The 5-hydroxymethylfurfural (HMF) content of the honey samples was determined by high-performance liquid chromatography (HPLC) under $OD_{285}$ detection, according to the AOAC method 980.23 [27]. Five grams of each honey sample were diluted to 50 mL and filtered through a membrane (0.45 µm). The HPLC analysis was performed using a Waters 1525 pumping system, Waters 2489 detector, RP-18 GP250 column (4.6 mm), and Waters 717plus autosampler. The isocratic mobile phase consisted of 90% water and 10% methanol at a flow rate of 0.7 mL/min. The HMF contents of the samples were calculated according to the corresponding peaks of the HMF standard solutions. A linear relationship ($R^2$ = 0.9981) was observed between the concentration and area of the HMF peak. Each sample was analyzed three times, and the mean HMF content was expressed as mg/kg.

### 2.3. Determination of Total Phenolic and Flavonoid Content of Honey

The honey samples' total phenolic content (TPC) was determined according to the method described by Wu et al [23]. Standard gallic acid (GA) and honey samples were diluted in a 60% acidified methanol solution containing 1% HCl. One hundred microlitres of the samples or standard were added to 2 mL of 2% $Na_2CO_3$ and allowed to equilibrate for 2 min. Then, 100 µL of 50% Folin-Ciocalteau reagent was added, and the solution was allowed to stand at room temperature for 30 min. Absorbance at 750 nm was measured at room temperature using an Ultrospec 2100 spectrophotometer (Biochrom, USA). The standard curve of GA over a concentration range of 5 µg/ mL to 25 µg/ mL is used to calculate the TPC of honey. The TPC was expressed as milligrams of GA equivalents/gram honey (mg GAE/g).

The total flavonoid content (TFC) in honey was determined using the Al $(NO_3)_3$ colorimetric method

[23]. One millilitre of the standard flavonoid quercetin and the diluted honey samples were added with 300 µL of 5% $NaNO_2$ and reacted for 5 min. Next, 300 µL of 10% $Al(NO_3)_3$ was added and reacted for 5 min, then neutralized by adding 4 mL of 4% NaOH solution for 10 min. Quantification was performed by reporting the absorbance at 510 nm in the calibration graph of standard quercetin (0–100 µg/mL). The results were expressed as milligrams of quercetin equivalents per gram (mg QEAC/g). The experiments to determine the TPC and TFC in honey were performed in triplicate.

## 2.4. Free Radical Scavenging Activity and Reducing Capacity of Honey

The water content of all honey samples was adjusted to 35% using distilled water. For the reducing capacity assays, honey samples were diluted to half with phosphate buffer (10 mM, pH 7.4). The reducing capacity of the honey samples was determined by measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity and ferric reducing antioxidant power (FRAP).[25]

The DPPH free radical-scavenging activity assay was performed according to the method described by Wu et al [23]. with slight modifications. In this method, 0.3 mL of the diluted honey sample was mixed with 0.3 mL of 1.0 mM DPPH radical solution and 2.4 mL of 99% ethanol, and then left at room temperature (25-28°C) for 30 min in the dark before measuring the absorbance at 517 nm using a Ultrospec 2100 spectrophotometer. Ascorbic acid (0.1 and 1.0 mM) was used as the positive control. Free radical-scavenging activity was calculated using the following equation: scavenging activity (%) = [1 - (absorbance of sample/absorbance of positive control)] × 100. All measurements were performed in triplicate.

The FRAP assay was performed according to the methods described by Wu et al., [23] with slight modifications. In this method, 180 µL of the FRAP reagent (300 mM sodium acetate buffer, pH 3.6; 10 mM 2,4,6-tripyridyl-striazine solution; and 20 mM FeCl3 solution) was mixed with 20 µL of diluted honey sample and incubated at 37°C for 4 min. The absorbance was then measured at 600 nm. The reducing ability of honey was expressed as mmol Fe2+ equivalents/kg honey. All measurements were performed in triplicate.

## 2.5. Preparation of PM2.5

Particulate matter (Standard Reference Material 1649b; SRM-1649b) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The PM2.5 sample used in this study is a standard urban dust sample (SRM 1649 B) containing polycyclic aromatic hydrocarbons (PAHs), nitro-substituted PAHs (nitro-PAHs), chlorinated pesticides, and inorganic constituents. The SRM 1649 B city dust suspension was prepared in LHC-9 cell culture medium at a concentration of 1000 µg/mL [28]. The suspended particles were vortexed and sonicated for 30 min before use to avoid the agglomeration of suspended PM2.5 and passed through a 0.22-µm filter. The cells were exposed to a PM concentration of 100 µg/mL for 24 h.

## 2.6. Cell Culture and MTT Assay

The human lung epithelial cell line BEAS-2B (ATCC® CRL9609™) was maintained in LHC-9 Medium (Gibco, USA) in a 37°C incubator (Astec, USA) in a humidified atmosphere containing 5% $CO_2$. Cells were treated with PM2.5 or honey samples for 24 h, and viability was measured by MTT assay according to the manufacturer's protocol [28]. The cells were seeded in 96-well plates at a density of $10^4$ cells per well. After incubation for 24 h, the cells were washed and incubated with PM2.5 or honey. Viable cells were stained with 2 mg/mL MTT solution (Thermo, USA) and incubated for 4 h. Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (TECAN, Switzerland) at room temperature.

## 2.7. Western Blotting

BEAS-2B cells were treated with PM2.5 and honey samples (10 µg/mL) for 24 h, washed three times with cold PBS, and lysed in 200 µL of protein extraction buffer (1 M Tris-HCl, pH 7.9, 3 M NaCl, 1% aprotinin, 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol) for 30 min on ice. The lysates were centrifuged at 12,000 × *g* for 20 min at 4°C, and supernatants were analyzed by western blotting. The lysate protein concentrations were measured using a BCA Kit (Visual Protein, Taiwan). Proteins (40 µg/lane) were electrophoretically separated by 12% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 2 h in 5% non-fat milk in tris-buffered saline. ZO-1, ZO-2, and AAT levels were measured using the respective cognate antibodies (1:1000). Secondary anti-mouse or anti-rabbit antibodies (1:1000) were coupled to horseradish peroxidase. MultiGel-21 (TOPBIO, JAPAN) was used to quantify chemiluminescence. Membranes were probed with stain-free total protein as the loading control [29].

## 2.8. Inflammatory Cytokine Assay

The levels of interleukin (IL)-8 in the culture medium supernatant after sample exposure were determined using an ELISA Development System Human CXI8/IL-8 Kit (R&D Systems, Minneapolis, MN, USA). The absorbance was recorded at 450 nm using an ELISA reader (TECAN, Switzerland).

## 2.9. Statistical Analyses

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Data expressed as the mean ± standard deviation (SD) for each experiment were analyzed using analysis of variance (ANOVA). The least significant difference test was used for pairwise comparisons when the ANOVA revealed significant differences.

# 3. Results

## 3.1. Physicochemical Parameters of the Honeys

The descriptive statistics for the physicochemical parameters of the honey samples, such as water content, sugar content, protein content, pH, total acidity, diastase activity, and HMF, are presented in Table 1. Water content depended on the processing technique and honeybee species. The Cerana1 and stingless bee honey samples were not concentrated on processing; therefore, they had a higher moisture content. Diastase activity was higher in the *A. mellifera* sample than in the samples from other bee species, and was not detectable in two stingless bee honey samples, i.e., *H. itama* and *T. binghami* honeys. The reducing sugar content (sum of glucose and fructose) of the honey samples was between 25.64%–69.15%, and was lower in the stingless bee honey samples. However, trehalulose was only obtained in the stingless bee honey samples (15.05%–17.84%). The protein content of the honey samples ranged from 331.19 ± 38.37 to 1098.98 ± 130.56 μg/g. The protein content in the honey samples differed according to the bee species and floral origin. The pH and total acidity in honey samples were 3.03–4.27 and 15.29–439.06 mEq/Kg, respectively. The honey collected from stingless bees showed low pH and high acidity.

## 3.2. Antioxidant Activity

Phenolic compounds and flavonoids are among the most important compounds that affect the antioxidant properties of honey [30,31]. Table 2 presents the data for the TPC, TFC, DPPH scavenging activity, and ferric reduction activity of the nine honey samples. TPC varied between 204.42 μg GAE/g (*H. itama* honey) and 1146.01 μg GAE/g (*A. dorsata* honey); no significant differences were observed in relation to bee species and botanical origin. The Cerana1 honey samples showed the lowest flavonoid content (7.96 μg QEAC/g), while the highest flavonoid content was identified in the *A. dorsata* honey (82.31 μg QEAC/g) followed by lychee honey (75.04 μg QEAC/g), multifloral honey (28.70 μg QEAC/g), longana honey (17.76 μg QEAC/g), Cerana2 honey (28.41 μg QEAC/g), *H. itama* honey (48.31 μg QEAC/g), *T. binghami* honey (28.70 μg QEAC/g), and *T. laeviceps* honey (74.92 μg QEAC/g); no significant differences were observed in relation to bee species and botanical origin.

The DPPH assay was used to determine the free radical-scavenging activity of the honey samples. In this study, the highest DPPH radical-scavenging activity (Table 2) was observed for longana honey (84.07%) and the lowest for lychee honey (55.13%). The FRAP assay was used to measure the ability to convert ferric to ferrous ion, that is Fe(III) to Fe(II), to further characterise the antioxidant activity (Table 2). Among the tested samples, the strongest reducing antioxidant activity measured by the FRAP assay was found for stingless bee *T. laeviceps* honey (5.28 ± 0.37 mmol $Fe^{2+}$/kg). The lowest reducing antioxidant power was detected in the Cerana2 honey sample (1.41 ± 0.31 mmol $Fe^{2+}$/kg). The results showed that differences in nectar plants and collection areas affected antioxidant activity more than the bee species.

**Table 1. Physicochemical parameters of different honey samples**

| | Fructose (g/100 g) | Glucose (g/100 g) | Trehalulose (g/100 g) | Moisture (%) | pH* | Total acidity (meq/Kg) | Diastase activity | HMF (mg/Kg) | Total Protein (μg/g) |
|---|---|---|---|---|---|---|---|---|---|
| Litchi honey | 31.19±0.12$^{e}$ | 34.52±0.09$^{a}$ | ND | 19.00± 0.10$^{a}$ | 4.22 | 15.29± 0.35$^{a}$ | 7.38±0.42$^{c}$ | 0.18±0.00$^{c}$ | 334.45±75.37$^{d}$ |
| Multifloral honey | 36.73±0.09$^{ab}$ | 31.01±0.07$^{c}$ | ND | 17.50± 0.20$^{b}$ | 4.07 | 26.87± 0.57$^{b}$ | 8.60±1.10$^{bc}$ | 0.10±0.00$^{d}$ | 780.30±152.62$^{bc}$ |
| Longan honey | 38.31±0.15$^{a}$ | 30.74±0.13$^{c}$ | ND | 19.20± 0.10$^{a}$ | 4.27 | 26.93± 0.61$^{b}$ | 13.58±1.47$^{a}$ | 0.08±0.00$^{e}$ | 1098.98±130.56$^{a}$ |
| Cerana1 honey | 33.34±0.01$^{d}$ | 30.21±0.11$^{c}$ | ND | 24.00± 0.10$^{c}$ | 4.12 | 68.29± 1.72$^{c}$ | 9.38±1.58$^{b}$ | 0.19±0.00$^{b}$ | 886.16±137.71$^{ab}$ |
| Cerana2 honey | 34.29±0.07$^{c}$ | 28.09±0.09$^{d}$ | ND | 19.50± 0.10$^{a}$ | 3.68 | 37.30± 1.11$^{d}$ | 3.32±1.41$^{d}$ | 0.55±0.00$^{a}$ | 446.43±86.50$^{c}$ |
| Dorsata honey | 31.34±0.12$^{e}$ | 33.46±0.15$^{b}$ | ND | 19.60± 0.10$^{a}$ | 3.17 | 68.45± 2.32$^{c}$ | 2.51±0.31$^{f}$ | 0.04±0.00$^{h}$ | 771.44±55.96$^{bc}$ |
| Itama honey | 21.06±0.09$^{g}$ | 22.44±0.09$^{e}$ | 17.84±0.01$^{ab}$ | 26.80± 0.20$^{d}$ | 3.33 | 142.43± 3.72$^{e}$ | ND | ND | 331.19±38.37$^{d}$ |
| Binghami honey | 12.53±0.11$^{h}$ | 13.11±0.07$^{f}$ | 17.43±0.03$^{a}$ | 36.70± 0.20$^{e}$ | 3.03 | 439.06± 6.72$^{f}$ | ND | ND | 382.62±53.08$^{d}$ |
| Laeviceps | 25.22±0.15$^{f}$ | 22.55±0.08$^{e}$ | 15.05±0.07$^{b}$ | 23.50± 0.10$^{c}$ | 3.03 | 78.74± 3.42$^{g}$ | 3.72±0.85$^{de}$ | 0.049±0.00$^{g}$ | 540.41±76.96$^{c}$ |

Means with different letters (a,b) within the different honey samples are significantly different ($P < 0.05$).
*The value was measured by using mixed three biological samples.

**Table 2. Antioxidant Activity of Various Honey Samples**

| | TPC (μg of GAE/g) | TFC (μg of QEAC/g) | DPPH Scavenging Activity (%) | Ferric reducing (mmol of $Fe^{2+}$/kg) |
|---|---|---|---|---|
| Litchi honey | 307.56±69.44$^{d}$ | 75.04±5.21$^{b}$ | 55.13±10.19$^{b}$ | 1.83±0.10$^{e}$ |
| Multifloral honey | 252.34±30.61$^{d}$ | 28.70±4.31$^{b}$ | 83.24±1.43$^{a}$ | 3.93±0.07$^{bc}$ |
| Longan honey | 329.66±38.59$^{d}$ | 17.76±7.45$^{b}$ | 84.07±3.47$^{a}$ | 1.58±0.15$^{e}$ |
| Cerana1 honey | 271.25±33.64$^{d}$ | 7.96±0.69$^{b}$ | 81.49±2.48$^{a}$ | 2.45±0.18$^{d}$ |
| Cerana2 honey | 354.30±112.70$^{d}$ | 28.41±4.48$^{b}$ | 82.90±2.59$^{a}$ | 1.41±0.31$^{e}$ |
| Dorsata honey | 1146.01±50.99$^{b}$ | 82.31±4.05$^{b}$ | 69.89±1.25$^{b}$ | 4.45±0.48$^{b}$ |
| Itama honey | 204.42±10.51$^{d}$ | 48.31±4.60$^{b}$ | 72.81±1.31$^{a}$ | 1.59±0.15$^{e}$ |
| Binghami honey | 302.78±25.47$^{d}$ | 81.46±13.13$^{b}$ | 80.40±3.03$^{a}$ | 2.64±0.34$^{d}$ |
| Laeviceps | 791.43±55.52$^{c}$ | 74.92±8.10$^{b}$ | 63.22±14.15$^{b}$ | 5.28±0.37$^{e}$ |

Means with different letters (a,b…) within the different honey samples are significantly different ($P < 0.05$).

### 3.3. Effects of PM2.5 and Honey on the Viability of BEAS-2B Cells

The MTT assay was performed to determine the toxicity of PM2.5 and honey samples. In comparison with the findings for the control group, incubation with 2 mg/mL PM2.5 for 24 h decreased the cell viability to 60.54% ± 3.37%. However, treatment with honey at concentrations of 0.1%, 0.01%, and 0.001% resulted in no cytotoxicity of BEAS-2B cells in the MTT assay (data not shown). These experiments were performed to determine the optimal concentrations and incubation time of PM2.5 and honey samples for further experiments. Thus, on the basis of these results, pre-treatment with honey at concentrations under 0.01% and incubation with 2 mg/mL PM2.5 were used in the following evaluations.

### 3.4. Alleviation of Inflammation Induced by PM2.5

As shown in Figure 1, in comparison with the negative control group, exposure to PM2.5 significantly increased the secretion of IL-8 (471.52 pg/mL) in BEAS-2B cells. Pre-treatment with honey samples decreased the secretion of IL-8 in BEAS-2B cells. All honey samples showed high activity in alleviating the inflammation induced by PM2.5.

### 3.5. Effect of Honeys on the Expression of the COPD Marker AAT

COPD can be caused by the oxidation of AAT, a neutrophil elastase inhibitor [32]. AAT is an important biochemical marker of early COPD. As shown in Figure 2a and 3a, in comparison with the negative control group, exposure to PM2.5 significantly reduced the levels of AAT in BEAS-2B cells. Pre-treatment with honey samples restored the expression of AAT up to 46.0%-135.7% in BEAS-2B cells. Thus, honey samples restored the activity of ZO-1 and ZO-2 in BEAS-2B cells, with stingless bee honey and *T. laeviceps* honey showing the greatest activity.

### 3.6. Attenuation of the Epithelial Barrier Dysfunction Induced by PM2.5

Epithelial cells are the first line of mucosal defence. ZO-1 and ZO-2 are important factors of the epithelial barrier, as shown in Figure 2b, Figure 2c, Figure 3b, and Figure 3c. In comparison with the negative control group cells, exposure to PM2.5 significantly reduced the levels of ZO-1 and ZO-2 in BEAS-2B cells. Pre-treatment of honey samples could restore the expression of ZO-1 and ZO-2 up to 46.8%-88.9% in BEAS-2B cells. In the stingless honey group, the *T. laeviceps* honey showed the greatest restorative effect on ZO-1 and ZO-2 in BEAS-2B cells. The restoration of ZO-1 and ZO-2 levels in the other honey groups did not differ significantly.

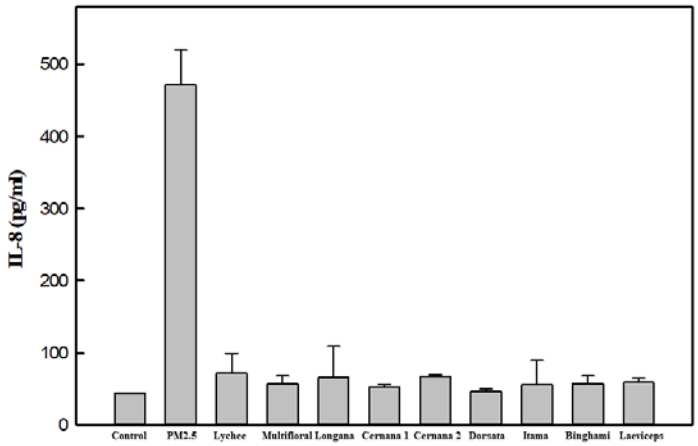


**Figure 1.** Interleukin-8 (IL-8) secretion titter of BEAS-2B cells after honey treatment. Data represent the mean of three repeats, with error bars indicating standard deviation. Bars with the symbol * are significantly different ($P < 0.05$) by the ANOVA test

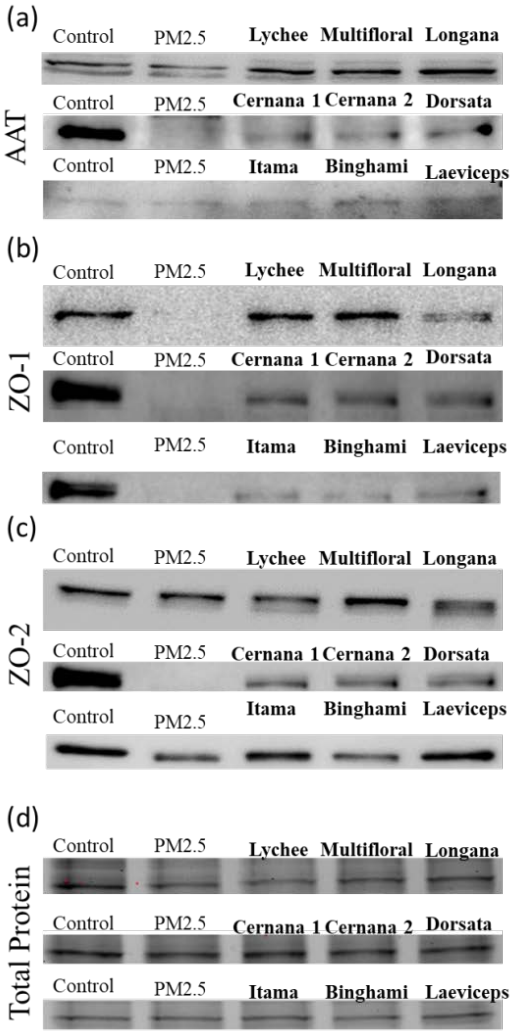


**Figure 2.** Western blot results of different honey samples protect on epithelial cells barriers disrupted by PM2.5 in BEAS-2B cells. (a) Expression of AAT. (b) Expression of ZO-1. (c ) Expression of ZO-2 (d) Free total protein staining as a loading control

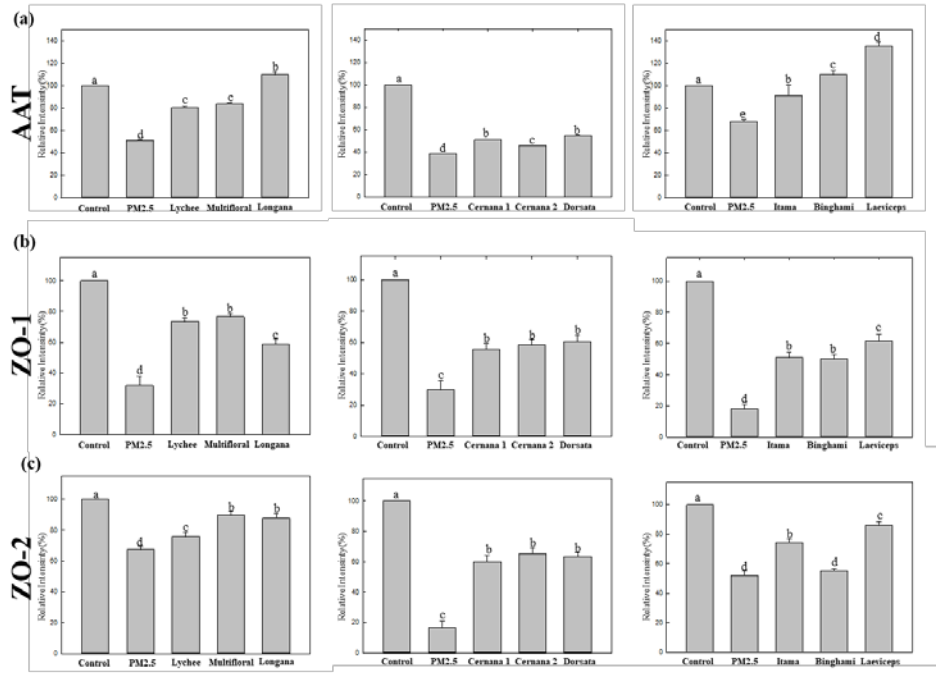


**Figure 3.** Effect of different honey samples on barrier function of epithelial cells disrupted by PM2.5 in BEAS-2B cells. Statistical results according to Figure 2, densitometric analysis was performed using NIH image analysis software for (A) Expression of AAT. (B) Expression of ZO-1. (C) Expression of ZO-2. Bars with different letters are significantly different ($P < 0.05$) by the ANOVA test

## 4. Discussion

In the present study, we unveiled, for the first, that honey could protective effects of honey against PM2.5-induced epithelial barrier damage and inflammation in BEAS-2B lung epithelial cells. Honey pre-treatment markedly inhibited PM2.5-induced risk factors for chronic obstructive pulmonary disease through anti-inflammatory activity and protection of the epithelial barrier. Our study proved the protective effects of honey against PM2.5-induced epithelial barrier damage and inflammation in BEAS-2B lung epithelial cells, which shed light on the preventing and protect PM2.5-triggered cell damage.

The composition, physicochemical properties, and biological activities of honey depend on various factors such as the harvesting season, flower source, type of honeybee, and environmental temperature [15,33]. All physiochemical parameters of honey obtained in this study fit the previously reported characteristics of *A. mellifera [15]*, *A. cerana* [22,29], *A. dorsata* [22,34], and stingless bee honeys [23,24,25]. Trehalulose, a beneficial disaccharide, is only found in stingless bee honeys [23,35]. It is a reducing sugar, in contrast to sucrose, and is present at high levels in stingless bee honeys. Stingless bee honeys are acidic (pH, 3.03 to 3.33), and their total acidity values range from 78.74 to 439.06 meq/kg, making them more acidic than *A. mellifera* and *A. cerana* honeys and the CODEX (2019) standard of 'not > 50 meq/kg' [36]. The total acidity value is often associated with honey fermentation; however, ions and organic acids can also contribute to a low pH and high total acidity. Stingless bee honeys have also been associated with high levels of total organic acids (e.g., such as gluconic acid, malic acid, succinic acid, oxalic acid, lactic acid, citric acid, and acetic acid) [37,38,39].

Among the many benefits of honey are its natural antioxidants, which are important for food preservation and human health because they combat the effects of oxidizing agents, reducing the incidence of heart disease, cancer, immune system decline, cataracts, and various forms of inflammation [40,41]. Various types of flower nectar and pollen collected by bees are responsible for honey's functional properties [42]. The antioxidant properties of honey can be attributed to the presence of phenolic acids, flavonoids, ascorbic acid, carotenoids, catalase, peroxidase, and Maillard reaction products [43,44]. Polyphenols are thought to exert their antioxidant activity primarily by neutralizing free radicals by donating electrons or hydrogen atoms [45]. The TPC and TFC of honey from different bee species are highly correlated with their floral origins [46]. Previous studies have indicated that the TPC range of honeys, including *A. mellifera*, *A. cerana*, *A. dorsata*, and stingless bee honeys, is 20–1965 μg GAE/g, while the TFC range is 9.1 to 224 μg QEAC/g [22]. In the present study, the values for all honey samples were within this range. The obtained results were comparable to those reported by Liu in Taiwan [15], where the DPPH activity varied from 15.24% to 69.3%. According to Tuksitha et al. [25] and Wu et al.,[23] the radical-scavenging activity of stingless bee honey samples ranges from 17.07%–86.6%. Moniruzzaman et al. [22] tested Malaysian honey produced by *A. cerana*, *A. dorsata*, and *A. mellifera* and found that the radical-scavenging activity measured for 10–60 mg/mL honey solutions ranges from 41.3% to 59.89%. DPPH activity and FRAP values are simple and direct tests that are widely used to determine the antioxidant activity of many different substances, including honey [15,22,23,25,47]. Floral origin strongly affects the DPPH free radical-scavenging activity and FRAP value of honey [15,48]. In this study, the floral and area sources mainly affected DPPH and FRAP, rather than the bee species.

Numerous studies have found that PM2.5 can cause oxidative stress, inflammation, metabolic disorders, and toxic mechanisms such as genotoxicity [49]. Oxidative stress and inflammation have been hypothesized to play roles in PM2.5-associated acute and chronic diseases including asthma, COPD, thrombus, atherosclerosis, autonomic nervous system disorders, skin aging, and type II diabetes [2,4,50]. Honey contains many active ingredients with antioxidant, anti-inflammatory, and other functional activities, making it a good complementary health source. Inflammation is another dominant factor responsible for PM2.5-induced toxicity, especially for the pulmonary diseases induced by PM2.5 exposure [51]. Previous studies have demonstrated that *A. mellifera* honey and stingless bee honey have good anti-inflammatory activities in intestinal cells [15,23]. In the present study, PM2.5 induced higher expression levels of IL-8 (Figure 1); however, pre-treatment with honey samples reduced IL-8 levels in BEAS-2B lung cells (Figure 1). All honey samples used in this study showed good anti-inflammatory activity.

Yang et al. described PM2.5-induced markers of oxidative stress through the AhR pathway that depletes ZO and AAT, suggesting that PM2.5 increases the risk of COPD [28]. ZO is a tight-junction protein that seals the paracellular pathway [52], and stimuli may disrupt its expression and the epithelial barrier. Yang et al. also investigated the ability of PM2.5 to disrupt the pulmonary barrier by depleting tight-junction proteins and impairing the normal function of AAT [28]. When tight-junction proteins are depleted, AAT is not effective at inhibiting neutrophil elastase, and excess neutrophil elastase accelerates elastin degradation in the lung parenchyma, thereby increasing the risk of COPD [14,28,32,53]. In the present study, honey pre-treatment was shown to distinctly restore the expression of ZO-1, ZO-2, and AAT in BEAS-2B cells after PM 2.5 treatment (Figure 2 and Figure 3). All honey samples exhibited similar activity trends. The present study demonstrates, for the first time, that honey pre-treatment protects BEAS-2B cells against injury by PM2.5. Thus, honey can reduce the risk of COPD through its anti-inflammatory activity and protect the epithelial barrier to reduce the damage caused by PM 2.5 to lung cells. The first line of defence against PM2.5 invasion is the pulmonary epithelial barrier, whose dysfunction is associated with altered pulmonary permeability and sensitivity to lung injury [54,55].

## 5. Conclusion

Honey has high nutritional and therapeutic value. The

phytochemical composition and antioxidant activities of honey are highly dependent on a variety of factors, including floral source, honey type, honey concentration, and bee type. In this study, we found that exposure to PM2.5 disrupted the barrier between lung epithelial cells by depleting the levels of ZO-1, ZO-2, and AAT. However, honey treatment protected lung epithelial cells against PM2.5-induced lung epithelial cell barrier disruption, inflammation, and injury. This study showed for the first time that honey supplements may protect against the harmful effects of PM2.5 on lung tissues, making them potential candidates for protection against pollution-induced lung damage.

## Authors' Contributions

C.C.P. and H.C.L. conceptualized the research; H.C.L. and H.Q.L. carried out data curation; Formal analysis by H.C.L., H.Q.L. and C.C.P; C.C.P. and M.C.W. contributed reagents and analytical tools; C.C.P. and M.C.W. supervised the study; C.C.P. and M.C.W. validated the data; H.C.L., M.C.W. and C.C.P. wrote the manuscript, conceived and designed research, reviewed and edited the draft. All authors read and approved the final published version of the manuscript.

## Funding

This work was supported by grants from the National Science and Technology Council, Taiwan, ROC (NSTC 112-2320-B-150-002) and Miaoli District Agricultural Research and Extension Station, Ministry of Agriculture,Taiwan ROC (112il01).

## ACKNOWLEDGMENT

We thank Mr. Ping-Hei Chen of YONG SHYANG HONEY ENTERPRISE Co., Ltd. for preparing honey samples.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

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