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Protective effects of *Mosla Chinensis Maxim* against Lung Injury

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Abstract	Original Research Article

Lung fibrosis is a common form of interstitial lung disease. Traditional Chinese medicinal plants have the ability to suppress lung inflammation, but there are relatively few studies on the relationship between *Mosla Chinensis Maxim* and lung injury. The purpose of this study was to investigate whether *Mosla Chinensis Maxim* extract (MCME) can alleviate lung injury. This study utilized lung epithelial cells, A549, treated MCME (0.25mg/mL), and analyzed the wound-healing ability, inflammatory cytokines, and cell-cell adheren junctions (E-cadherin). Bleomycin was used to induced lung fibrosis in an animal model, which were then treated with MCME. The results showed that MCME improved cellular repair capacity by 30% compared to the mock group, inhibited IL-8 by approximately 60%, and increased the E-cadherin expression compared to the lipopolysaccharide group. In an animal model, administration of MCME (50 mg/kg) for 14 days alleviated the bleomycin-induced pathological changes in the lungs and fibrosis. This study demonstrated that MCME improved lung injury.
Keywords: Chinese herbalplants*, Mosla Chinensis Maxim,* Lung fibrosis, Inflammation.

1. INTRODUCTION

Injuries to the lungs are a grave health issue that can be caused by various conditions including sepsis, trauma, pneumonia, or inhaling toxic substances from the air. These conditions can result in damage to the structure or function of the lungs, causing symptoms such as breathing difficulties, shortness of breath, coughing, chest pain, chest tightness, and anxiety in affected patients [1, 2]. Lung injury is characterized by several clinical features, including decreased lung volume, impaired lung function, and an altered ratio of blood flow to ventilation that cannot be accurately measured [1]. In more severe cases, lung injury can lead to respiratory distress syndrome, characterized by bilateral infiltration of the lungs and ultimately resulting in respiratory failure [3]. Lung injury typically involves damage to the alveolar epithelial cells, resulting in various types of edema such as pulmonary, interstitial, or alveolar edema, acute hypoxia, and the infiltration of inflammatory cells, particularly leukocytes [4]. The infiltration of leukocytes, especially neutrophils, plays a crucial role in initiating inflammatory responses in lung injury [5]. Animal studies have shown that lung injury is associated with an increase in cytokines, which leads to

a reduction in antioxidants, up regulation of adhesion molecules on pulmonary vascular endothelial cells, and an increased susceptibility to injury of these endothelial cells.[6]. A study indicated that inhibition of the inflammatory response in lung epithelial cells may alleviate lung injury induced by lipopolysaccharide (LPS) [7]. The over expression of Bcl-2 and the knockdown of Bad were both found to mitigate LPS-induced lung injury and prevent apoptosis in lung epithelial cells [8]. To ensure proper lung function, it is essential to minimize exposure to air pollutants and pathogens as they can cause lung tissue damage and contribute to pulmonary fibrosis. [9]. Despite efforts to inhibit lung injury progression, there are limitations to the degree of success achieved, making it crucial to identify novel strategies for prevention. With an increasing number of plants being used for medicinal purposes that can potentially inhibit inflammatory responses, there is a growing need to conduct more studies related to lung injury [10].

Mosla Chinensis Maxim (MCM) is a cultivar of the wild-type M. chinensis plant, which belongs to the Labiatae family and is found widely distributed in

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countries such as China, Vietnam, India, and Japan [11]. The leaves of Mosla Chinensis Maxim are commonly used as a wild vegetable or as a flavoring agent in foods due to their aroma and taste. In traditional Chinese medicine, the extract of Mosla Chinensis Maxim (MCME) is used to treat a wide range of ailments including cold, fever, diarrhea, dysentery, digestive disorders, vomiting, stroke, and edema [12]. The active compounds in Mosla Chinensis Maxim extract (MCME) have been documented to possess a range of health-promoting properties, including antibacterial, antiviral, antioxidant, anti-inflammatory, analgesic, antipyretic, analgesia, and immune-regulatory effects [13]. As of now, the therapeutic potential of Mosla Chinensis Maxim extract (MCME) in treating lung disease is still unclear. However, studies have shown that Bleomycin, a commonly used chemotherapeutic drug for treating different types of human carcinomas, can cause pulmonary injury and fibrosis in both humans and experimental animal models [14]. Despite the significant advances in medical strategies made over the past few decades, there is still a lack of Chinese herbal medicines available for the prevention and treatment of lung injury [15]. Therefore, our study aimed to evaluate whether Mosla Chinensis Maxim extract (MCME) can protect against lung injury and to determine the possible underlying mechanisms of action.

2. METHODS
2.1 Isolation of MCME
MCM was purchased from Hunan, China, and included the whole plant, including the stems, leaves, and flowers. The whole plant crushed and combined with water to a 15:1 ratio (water:MCM powder,wt:wt).This was extracted at 55°C±5°C for about 30 min to the Brix degree of the solution. When the value (Degrees Brix) was greater than 0.7, the extraction is halted to form a first extract containing solids, and the extract was filtered through a 400-mesh filter to remove the fine solids. A concentrator (brand/model: BUCHI -Rotavapor R-100) was used to concentrate the aforementioned extract under reduced pressure conditions at 70°C±5°C until the Brix value of the solution (Degrees Brix) was 10±0.5 and concentrating was stopped to obtain the final MCM extract.

2.2 Cell Culture
A549 lung epithelial-like cells were cultured in RPMI 1640 culture medium supplemented with GlutaMAX™ (Gibco/Life Technologies, cat#: 61870-010), 10% fetal bovine serum (FBS, Gibco/Life Technologies, cat#: 10270-106), and 1% penicillin-streptomycin (Gibco Life Technologies, cat#: 15140-122) at 37°C and 5% CO2. The cells were seeded at a density of 5×104 cells/cm2 on the apical side of the alveolar membrane and the cell culture medium was changed daily. Once the cells reached confluence after 48 hours, they were cultured in a starvation medium (RPMI 1640 without FBS) for an additional 24 hours.

2.3 Wound-Healing Assay
The study utilized a wound-healing assay to evaluate the wound-healing capacity of the A549 lung epithelial- like cells. The cells were seeded in 6-well plates at a density of $5×10^4$ cells per well. A wound was created in the epithelial cell monolayer by dragging a 10 μL pipette tip across the thin membrane. After washing the cells once with starvation medium to remove cell debris, the area of the scratch was recorded daily by capturing images under a phase contrast microscope. The images of the same fields of view were captured, and the gap width was measured. Each experiment was performed in triplicate or quadruplicate and repeated at least twice.

2.4 Immunofluorescence Assay
To observe the tight junctions, the cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) and then immunostained with goat anti–E-cadherin antibody (Abcam, cat#: ab40772). To block non-specific binding, the cells were treated with 2% FBS in phosphate buffered saline. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using a Zeiss Axioplan microscope with a 20× objective.

2.5 Cytokine ELISAs
To measure the release of IL-8, a direct sandwich enzyme-linked immunosorbent assay (ELISA) was performed. Initially, immunoassay plates were coated with mouse monoclonal anti-human IL-8 antibodies at a concentration of 4 μg/mL. After a blocking step, samples were added to the plates. To obtain the standard titration curves, recombinant human IL-8 was used. Next, biotinylated anti-IL-8 antibodies were added at a concentration of 40 ng/mL to sandwich the antigen. The plates were then incubated with horseradish peroxidase (HRP)-conjugated streptavidin and substrate (H2O2 and ABTS) was added. Absorbance was measured at 450 nm using a microplate reader, and the data was analyzed using SLT LabInstruments software. The IL-8 ELISA had a sensitivity of 15 pg/mL.

2.6 Animal Model
This study was conducted in compliance with the US National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Fu Jen Catholic University in Taiwan. Male C57BL/6 mice, aged eight weeks, were housed under controlled temperature (21°C±2°C) and a 12-hour light-dark cycle with ad libitum access to food and water. The mice were randomly divided into three groups: control, lung fibrosis, and MCME-treated lung fibrosis. Pulmonary fibrosis was induced by intratracheal injection of bleomycin. Mice were anesthetized with 2% isoflurane mixed with 1 L/min O2 in an induction chamber. After anesthesia, a midline incision was made in the neck skin, and the trachea was exposed by dissection. The trachea

was then injected with bleomycin dissolved in sterile saline using a 1 mL syringe. The mice were euthanized 14 days after bleomycin administration, and their lungs were excised. To evaluate the effect of MCME on pulmonary fibrosis, MCME was orally administered at a dose of 50 mg/kg daily, starting from two days before bleomycin injection-induced pulmonary injury and continuing until the end of the study.

2.7 Lung Histology
The lungs were fixed in 4% PFA, embedded in paraffin, and horizontally sectioned into 4 μm slices. Masson's trichrome staining was carried out to identify collagen fibers. Pathohistological scores were assigned to each field individually to evaluate the severity of the lung injury.

2.8 Micro-Computed Tomography (micro-CT) Imaging
The lungs were first fixed in 4% PFA and then soaked in ethanol overnight. After that, the lungs were placed in 100% hexamethyldisilazane for 2 hours before being prepared for micro-CT scanning. The Skyscan 1272 was used to perform the scanning at a resolution of

10.0 μm. The entire lung was captured during the scan, and any areas with fibrosis were highlighted in red.

2.9 Statistical Analysis
All values are represented as mean ± standard error. The results were analyzed using analysis of variance, followed by Bonferroni post hoc tests. $P<0.05$ was considered significant.

3. RESULTS
3.1 MCME-Improved Lung Injury *in Vitro*
To examine whether MCME improved lung injury *in vitro*, we used 10 ng/mL LPS to induce acute lung injury with or without MCME (25 mg/mL) treatments in A549 cells. MCME improved the lung epithelial cell repair capacity up to 30% compared to the mock group (Figure 1A). MCME inhibited the secretion of IL-8, which is induced by LPS, by up to 60% compared to the LPS group (Figure 1B), and recovered the expression of the epithelial marker E-cadherin compared to the LPS group (Figure 1C). These results showed that MCME improved self-repair and prevented lung injury.
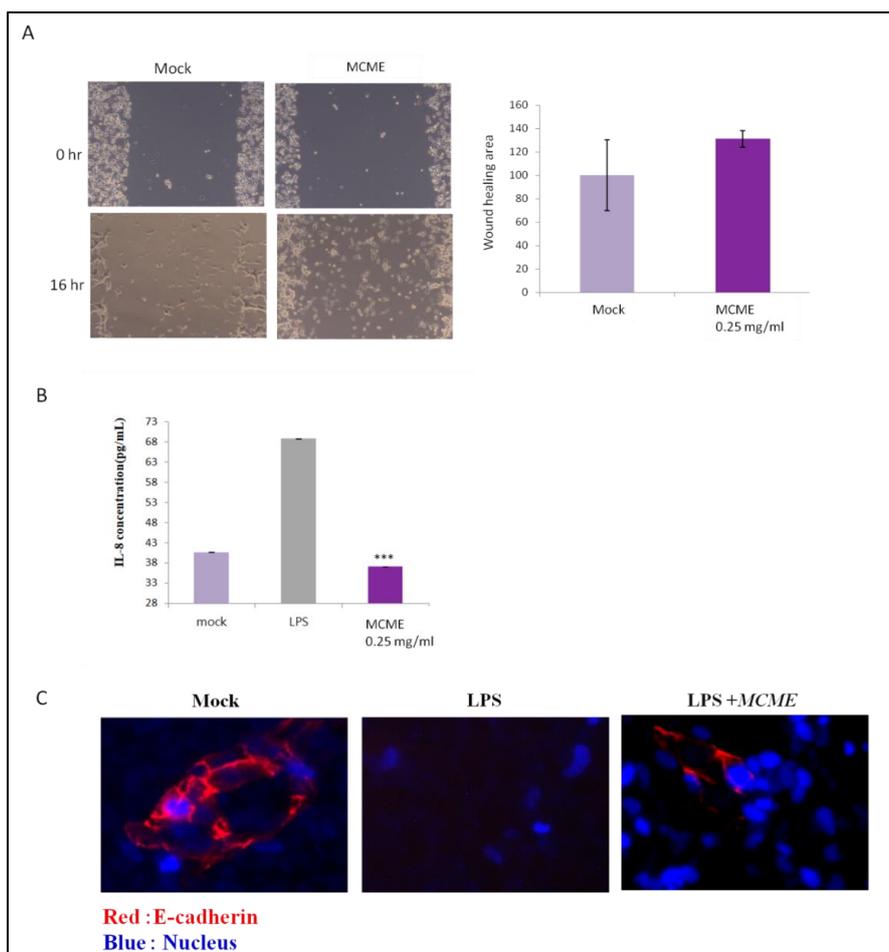

Figure 1: Effect of MCME on lung epithelial cells. (A) Effect of MCME on lung cell migration as assessed by a wound-healing assay. Representative digital pictures were taken at 0 and 16 h. (B) IL-8 production after pre-incubation of lung cells with LPS (10ng/mL) or MCME (0.25 mg/mL) for 24h by ELISA assay. (C) The expression of E-cadherin was observed at the cell-cell adherens junctions by immunofluorescence assay. Data are represented as mean ± standard error of two independent experiments

3.2 MCME Suppressed Lung Injury in Mice

To examine whether MCME alleviated lung injury *in vivo*, eight-week-old male C57BL/6 mice were randomly divided into three groups. Thickening of the alveolar wall and collagen deposition were found in the lungs of the mice treated with bleomycin (Figure 2A) compared to the normal morphology of the control group. Bleomycin administration resulted in higher histopathological scores, compared with the control group, whereas MCME treatment alleviated the bleomycin-induced pathological changes in the lungs (Figure 2B).
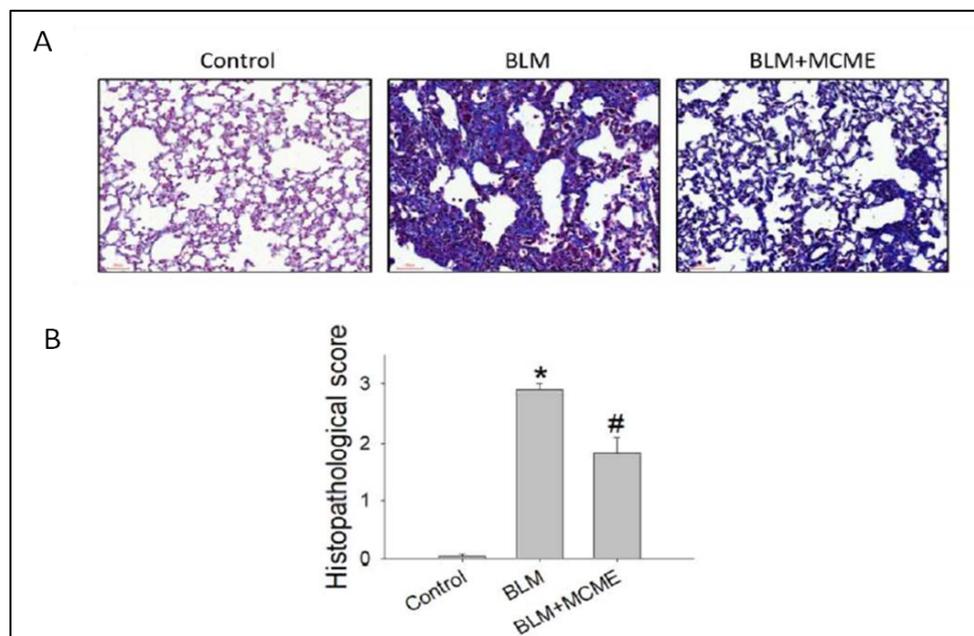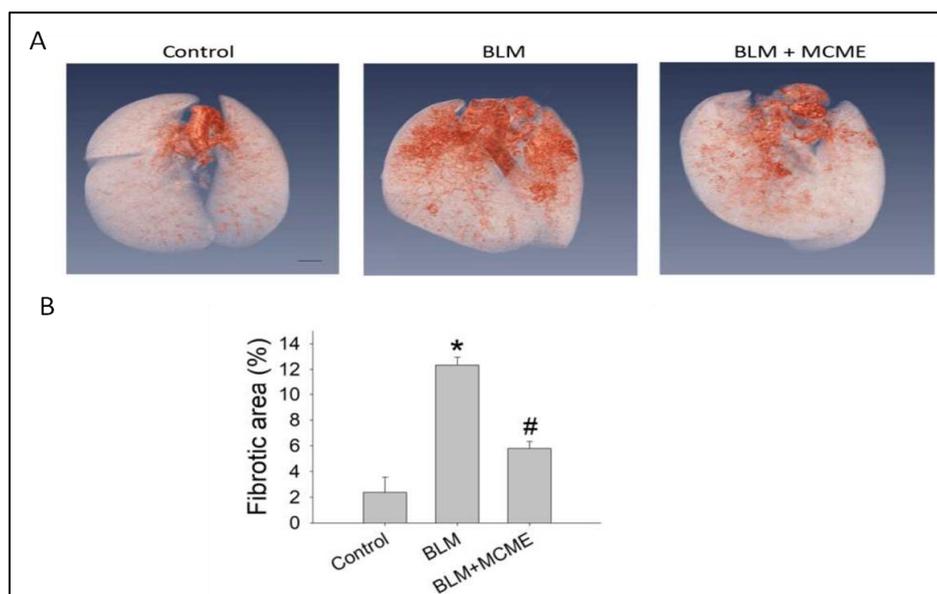

Figure 2: MCME ameliorated the morphological changes in lung injury. Mice were given intratrachealbleomycin (BLM) and sacrificed after 14 days. *MoslaChinenesis Maxim* extraction (MCME; 50 mg/kg) was orally administered daily, from 2 days before BLM injection-induced lung fibrosis, until the end of the study. Lung tissues were collected and stained with Masson's trichrome staining. (A) Representative histological sections of the lungs are shown. Blue staining denotes the regions of injury. (B) Histopathological score of lung section. (n=6) *P<0.05 vs. control, #P<0.05 vs. BLM

3.3 MCME Alleviated Lung Fibrosis in Mice

Micro-CT was performed to detect the fibrotic areas in the whole lung (Figure 3A). MCME significantly ameliorated the bleomycin-induced lung fibrosis (Figure 3B). These results indicate that MCME relieved lung fibrosis induced by bleomycin.

Figure 3: MCME suppressed BLM-induced lung fibrosis. Mice were given intratrachealbleomycin (BLM) and sacrificed after 14 days. *MoslaChinenesis Maxim* extraction (MCME; 50 mg/kg) was orally administered daily, from 2 days before BLM injection-induced lung fibrosis, until the end of the study. Lung tissues were collected for micro-CT analysis. (A) Representive 3D volume reconstructions of the lungs are shown. Lung fibrotic area is labeled in red. (B) Percentage of lung fibrosis of the whole lung. (n=4) *P<0.05 vs. control, #P<0.05 vs. BLM

4. DISCUSSION

In lung fibrosis, the cellular composition of the alveolar region is altered, and there is an abnormal accumulation of collagen [16, 17]. This respiratory condition is a chronic and progressive lung disease that is complex and debilitating, with a bleak prognosis that often leads to severe breathing difficulties [17, 18]. Due to the limited understanding of the fundamental mechanisms underlying the pathogenesis of this lethal and progressive disease, treatment options are currently limited [17, 19]. This study provides the first evidence that MCME has the potential to enhance lung self-repair mechanisms and prevent the development of pulmonary fibrosis. The pathogenesis of lung fibrosis is multifaceted and involves intricate interactions between cells responsible for regeneration and repair. A hallmark feature of fibrotic lung tissue is the accumulation of extracellular matrix [20]. The buildup of extracellular matrix is a common feature of fibrotic tissue [20, 21]. In this study, the administration of MCME was found to decrease the levels of collagen in the lung tissue, which is a key indicator of the histological signs of lung fibrosis.

Emerging evidence suggests that alterations in the physical structure of the epithelium play a crucial role in the early development of numerous chronic lung diseases. E-cadherin, synthesized by epithelial cells, constitutes the main structural component of apical junctional complexes. In the absence of E-cadherin expression, tight junction proteins such as zonula occludens-1 (ZO-1), occludin, and claudins fail to form correctly [22]. In another study found that reducing E-cadherin expression through siRNA knockdown resulted in decreased expression of ZO-1 and lowered epithelial resistance [23]. Consistent with our results, MCME recovered E-cadherin expression, suggesting that MCME improved the lung physical structure after injury. Several factors play important roles in the development of fibrosis, such as cytokines, chemokines, eicosanoids, fibrinogenic factors, oxidative stress, matrix metalloproteinases, and their inhibitors [24]. Examination of lung tissue through histological evaluation revealed additional indications of inflammatory disorders in individuals suffering from pulmonary fibrosis [24]. The abnormal accumulation of inflammatory cells, such as alveolar macrophages and neutrophils, is thought to be the underlying cause of the chronic inflammatory response associated with this disease [21, 25]. During the development of lung fibrosis, immune cells accumulate and release cytokines that stimulate the migration of differentiated mesenchymal cells into areas of lung injury. These mesenchymal cells are then stimulated to secrete collagen, leading to abnormal collagen deposition and fibrotic tissue formation [21]. The pathogenesis of lung fibrosis has been strongly associated with cytokines released from the activated alveolar immune cells [25]. Thus, the management of pulmonary fibrosis and other lung disorders has primarily emphasized the improvement of underlying causative factors, including inflammation. The upregulation of pro-inflammatory cytokines such as TNF-α, IL-8, and IL-6 in lung tissues serves as significant markers of pulmonary inflammation [25, 26]. Therapeutic use of plant extracts has the potential to regulate the secretion of various cytokines involved in respiratory diseases such as IL-1β, IL-8, IL-17, IL-10, and IL-23, and can also modulate the levels of airway inflammatory biomarkers. Therefore, plant extract therapy may have the ability to control respiratory diseases [27]. The Toll-like receptor (TLR) family comprises crucial receptors in the innate immune and inflammatory systems [28]. Upon recognition of pathogens, these receptors activate intracellular signaling pathways [28, 29]. Myeloid differentiation primary response 88 (MyD88) is a crucial adaptor molecule in the TLR signaling pathway, serving as a key mediator in transmitting upstream information and influencing disease progression [29]. NF-κB is a crucial transcription factor involved in the immune and inflammatory responses of cells downstream of the TLR signaling pathway [29]. Treatment with MCME was found to reduce the expression levels of TLR, MyD88, and NF-κB, which are important components of the TLR signaling pathway [30]. Based on the findings of this study, it is hypothesized that the protective effect of MCME in lung fibrosis may be attributed to its anti-inflammatory activity. However, further research is needed to fully understand the underlying mechanism.

Abundant substances such as volatile oil, flavonoids, and other active constituents have been identified in MCM [31]. A previous study reported that the main flavonoids present in MCME were luteolin and apigenin, both of which have been shown to have anti-influenza properties [30]. The polysaccharides and flavonoids found in MCME have been shown to boost immunity, possess antioxidant properties, and exhibit antibacterial activity in mice [32]. The neutral polysaccharides extracted from MCME have been shown to exhibit antioxidant and immunomodulatory activities when isolated [33]. Based on our previous experiments (unpublished data), it was found that MCME has a high content of rosmarinic acid. Rosmarinic acid is a type of polyphenol found in nature that possesses antioxidative and anti-inflammatory properties. In a previous study, it was found that rosmarinic acid can prevent diesel exhaust particle-induced lung injury by reducing the expression of proinflammatory molecules [34]. Previous studies have suggested that rosmarinic acid possesses antioxidative properties, which are attributed to its ability to scavenge free radicals through its catechol hydroxyl group, as well as regulate the levels of NADPH oxidases and superoxide dismutases [35]. Further investigation is necessary to identify the key active compounds and mechanisms responsible for regulating lung injury.

5. CONCLUSIONS

The results of this study demonstrate that MCME treatment can enhance lung epithelial cell repair

and reduce inflammation, ultimately leading to the inhibition of lung epithelial cell injury and a decrease in bleomycin-induced lung fibrosis. These findings support the potential therapeutic applications of MCME in the prevention of lung injury.

Ethics Statements

This study was conducted in compliance with the US National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Fu Jen Catholic University in Taiwan.

Credit Authorship Contribution Statement

Hui-Chun Ku: Data curation, Investigation, Methodology, Writing – original draft. Shih-Yi Lee: Formal analysis, Software, Writing – review & editing. Yung-Kai Lin: Resources. Yung-Hsiang Lin: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. Chi-Fu Chiang: Conceptualization, Funding acquisition, Project administration, Writing – review &editing.

DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest.

Data Availability

Data will be made available on require.

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