Aqueous *Thunbergia laurifolia* leaf extract alleviates paraquat-induced lung injury in rats by inhibiting oxidative stress and inflammation

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**Abstract**

**Background:** Paraquat (PQ) has been reported to have a high mortality rate. The major target organ of PQ poisoning is the lungs. The pathogenesis of PQ-induced lung injury involves oxidative stress and inflammation. Unfortunately, there is still no effective antidote for PQ poisoning. We hypothesized that aqueous *Thunbergia laurifolia* (TL) leaf extract is a possible antidote for PQ-induced lung injury.

**Methods:** The total phenolic content and caffeic acid content of an aqueous extract of TL leaves were analyzed. Male Wistar rats were randomly divided into four groups (n = 4 per group): the control group (administered normal saline), the PQ group (administered 18 mg/kg body weight (BW) PQ dichloride subcutaneously), the PQ + TL-low-dose (LD) group (administered PQ dichloride subcutaneously and 100 mg/kg BW aqueous TL leaf extract by oral gavage) and the PQ + TL-high-dose (HD) group (administered PQ dichloride subcutaneously and 200 mg/kg BW aqueous TL leaf extract by oral gavage). Malondialdehyde (MDA) levels and lung histopathology were analyzed. In addition, the mRNA expression of NADPH oxidase (NOX), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) was assessed using reverse transcription-polymerase chain reaction (RT-PCR), and the protein expression of IL-1β and TNF-α was analyzed using immunohistochemistry.

**Results:** The total phenolic content of the extract was 20.1 ± 0.39 μg gallic acid equivalents (Eq)/mg extract, and the caffeic acid content was 0.31 ± 0.01 μg/mg. The PQ group showed significantly higher MDA levels and NOX, IL-1β and TNF-α mRNA expression than the control group. Significant pathological changes, including alveolar edema, diffuse alveolar collapse, hemorrhage, leukocyte infiltration, alveolar septal thickening and vascular congestion, were observed in the PQ group compared with the control group. However, the aqueous TL leaf extract significantly attenuated the PQ-induced increases in MDA levels and NOX, IL-1β and TNF-α expressions. Moreover, the aqueous TL leaf extract ameliorated PQ-induced lung pathology.

**Conclusion:** This study indicates that aqueous TL leaf extract can ameliorate PQ-induced lung pathology by modulating oxidative stress through inhibition of NOX and by regulating inflammation through inhibition of IL-1β and TNF-α expressions. We suggest that aqueous TL leaf extract can be used as an antidote for PQ-induced lung injury.

**Keywords:** Paraquat, *Thunbergia laurifolia*, Lung injury, Oxidative stress, Inflammation, Malondialdehyde, NADPH oxidase, Interleukin 1 beta, Tumor necrosis factor alpha

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Background
Paraquat (PQ) is a bipyridylium herbicide that has been shown to be severely toxic to humans. PQ exposure, either accidental or intentional, is associated with high mortality rates. The incidence rate of PQ intoxication is at least 3.8 cases per 100,000 inhabitants annually [1]. The ingested amount of PQ is the key factor affecting the prognosis of patients [1]. The 50% lethal dose (LD$_{50}$) in humans is approximately 35 mg/kg [2]. PQ accumulates mainly in the lungs, specifically in type I and type II pneumocytes and Clara cells, at 6–10 times the levels in plasma [3]. The lung pathology associated with PQ poisoning involves destructive and proliferative phases [4]. The destructive phase comprises alveolar epithelial swelling and fragmentation, alveolar edema and acute inflammation, while the proliferative phase involves diffuse intra-alveolar fibrosis via promotion of fibroblast infiltration into the alveolar space. In previous study, histological analysis revealed that the lungs of 11 patients who died from PQ poisoning demonstrated hemorrhage, macrophage extrusion, edema, honeycombing, fibrosis and, rarely, epithelial hyperplasia [5].

Oxidative stress, reduced antioxidant capacity and inflammation are key mechanisms of PQ-induced lung injury [6]. Oxidative stress results from imbalance between the generation and clearance of oxidants. PQ induces oxidative stress by inducing the production of large amounts of reactive oxygen species (ROS), including superoxide radicals (O$_2^-$), hydroxyl radicals (‘OH), and hydrogen peroxide (H$_2$O$_2$), via redox cycling [7]. ROS are highly reactive with cellular macromolecules such as lipids, proteins and nucleic acids, and ROS reactions cause lipid peroxidation, protein carbonylation and DNA damage. NADPH oxidase (NOX), which is widely distributed in several tissues and organs, typically catalyzes the reduction of molecular oxygen (O$_2$) to produce O$_2^-$ [8]. Notably, high levels of PQ cause ROS-mediated inflammation by enhancing the levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) [9].

Currently, there is still no proven antidote or effective treatment for PQ poisoning. However, novel compounds with antioxidant and/or anti-inflammatory properties have been explored, especially traditional herbal medicines, because of their efficacy and safety. A traditional Thai herb, Thunbergia laurifolia (TL), exhibits various biological properties, particularly curative, anti-inflammatory and antioxidant properties. TL has been widely used as a treatment agent for poisoning caused by toxic substances such as drugs, alcohol and heavy metals [10, 11]. TL aqueous extract at 25 mg/kg (orally) for 7 days showed the hepatoprotective activity against ethanol induced liver injury both in vitro and in vivo [12]. TL leaf extract at 100 mg/kg or 200 mg/kg body weight (orally) once a day can alleviate neuronal cell death and memory loss and restore via antioxidant activities in mice [10]. Wistar rats have been widely used to determine pharmacological effects of TL against toxicants [13, 14]. Therefore, the present study aimed to investigate the protective effect of an aqueous TL leaf extract against PQ-induced lung injury in male Wistar rats.

Methods
Preparation of aqueous extract of TL leaves
Leaves of TL were obtained from Nakhon Si Thammarat Province, Thailand (voucher specimen Thunbergia laurifolia AHS2008120101) and had been deposited at the Herbarium of Plant Genetic Conservation Project under The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG), Walailak University, Nakhon Si Thammarat. The leaves were washed and dried at 60°C in an oven and ground into powder using a blender. The leaf powder (10 g) was boiled in 100 ml of distilled water for 48 min. The filtrate was lyophilized in a freeze dryer (Eyela, Tokyo, Japan) and preserved at −80°C until use.

Analysis of total phenolic content
The content of phenolic compounds in aqueous extract of TL was determined using Folin-Ciocalteu reagent, which the method was described previously [15]. The working solution was made by dissolving the TL extract (25 mg) was dissolved in 2 ml water, filtered (a 0.45 μm filter), and diluted to various concentrations as a working solution. HPLC-UV analysis was performed using a Thermo Scientific Dionex Ultimate 3000 model (Thermo Scientific, MA, USA), which consisted of a variable wavelength detector (VWD-3100), an autosampler (WPS-3000SL), a tertiary pump (LPG-4300SD), and a column compartment (TCC-3000SL). Sample solution or caffeic acid standard (10μl)
was injected into a C18 analytical column (VertiSep™ HPLC columns, 250 mm × 4.6 mm, 5 μm particle size; Vertical Chromatography Co., Ltd., Nontaburi, Thailand). The HPLC conditions modified from those used in the previous study [16]. The mobile phases were composed of 60% (v/v) acetonitrile in water (solvent B) and 1% (v/v) acetic acid in water (solvent A). The flow rate was 0.7 ml/min, and the column was eluted with a linear gradient program of 30–40% solvent B over 0–7 min, 40–70% solvent B over 7–10 min, and 70–100% B over 10–28 min. The mobile phase was returned to 30% solvent B and maintained for 3 min to equilibrate the column before the next injection. The column compartment temperature was set to 30°C, and the eluted compounds were monitored at 330 nm. The column pressure was not precisely controlled during HPLC analysis. Instead, it was varied according to the gradient compositions of the mobile phase when flow rate of mobile phase was fixed. The analytical system operates at a pressure of between 100 and 140 bar, approximately. In response to the increased concentration of acetonitrile in the mobile phase, the pressure is reduced. The analysis was performed in triplicate.

**Experimental animals and treatments**

The study was performed in a manner of reduced the animal numbers and minimized animal suffering. Sixteen male Wistar rats (*Rattus norvegicus*) aged 6 weeks were obtained from Nomura Siam International Co, Ltd. (Bangkok, Thailand). The rats were maintained under constant temperature (23 ± 2°C) and relative humidity (50–60%) with a 12 h light/dark cycle. Food and water were provided ad libitum. The animal experimental procedures were performed in a way that adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

The rats were randomly divided into 4 groups (4 rats per group): the control group, in which rats received subcutaneous injection of 1 ml/kg body weight (BW) normal saline once a week for 6 weeks; the PQ group, in which rats received subcutaneous injection of 18 mg/kg BW PQ dichloride once a week for 6 weeks; the PQ+TL-LD group, in which rats received subcutaneous injection of 18mg/kg BW PQ dichloride and oral gavage of low-dose TL leaf extract (100 mg/kg BW) once a week for 6 weeks; and the PQ+TL-HD group, in which rats received subcutaneous injection of 18mg/kg BW PQ dichloride and oral gavage of high-dose TL leaf extract (200 mg/kg BW) once a week for 6 weeks. PQ and TL treatment was performed according to the procedures of Orito et al. [17] and Tangpong and Satarug [10]. The rats were euthanized by thiopental sodium overdose (100 mg/kg BW) anesthesia. The abdominal cavities were then opened, and the lungs were excised.

**Determination of malondialdehyde (MDA) levels**

MDA measurement was carried out using an OxiSelect™ TBARS Assay Kit (cat. no. STA-330, Cell Biolabs, Inc., USA) in accordance with the manufacturer’s protocol. Lung homogenate (50 mg/ml) was prepared by homogenizing lung sections on ice in phosphate-buffered saline containing 1 × butylated hydroxytoluene. The homogenate was centrifuged at 10,000×g for 5 min, and the MDA content was assayed [18].

**Histopathology**

Lung tissues were fixed in a 10% neutral buffered formalin solution, processed, and embedded in paraffin. The tissues were sliced into sections using a rotary microtome and subjected to hematoxylin and eosin (H & E) staining. The severity of pathological alterations was graded using the following semiquantitative scale: 0 = normal, 1 = pathological alterations in up to 25% of the high-power field, 2 = pathological alterations in 26–50% of the high-power field, 3 = pathological alterations in 51–75% of the high-power field, and 4 = pathological alterations in > 75% of the high-power field.

**Determination of pulmonary NOX, IL-1β and TNF-α mRNA expression using reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from lung tissue using a Tissue Total RNA Mini Kit (Geneaid, Korea). The purity and quantity of RNA were determined using a NanoDrop™ one/oneC Microvolume UV-Vis Spectrophotometer with Wi-Fi (Thermo Scientific, USA). RT-PCR was performed to amplify the genes. The thermal cycling conditions included an initial denaturation step at 95°C for 15 min, denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min, extension at 72°C for 1 min, and a final elongation step at 72°C for 10 min. The primers used are shown in Table 1. The DNA samples were loaded into a 2% agarose gel. After staining with ethidium bromide, the gel was visualized with a camera. The gene expression was calculated relative to the control group.

**Table 1 Primers of NOX, IL-1β, TNF-α and β-actin**

| Gene   | 5′-3′ Primer sequence        |
|--------|------------------------------|
| NOX    | Forward primer GGAATAAGAAAGTTCACCTGCC |
| IL-1β  | Reverse primer GTATGAGTGGCCATCCAGACAG |
| TNF-α  | Forward primer CCCGCAGGTGGAGATGGG |
| β-Actin| Forward primer GTAAGTGGCTACATGGCTGG |

The primers used were shown in Table 1. The DNA samples were loaded into a 2% agarose gel. After staining with ethidium bromide, the gel was visualized with a camera. The gene expression was calculated relative to the control group.
UV transilluminator. The amount of PCR product was detected using GeneTools software via image analysis (Syngene, Frederick, MD, USA).

**Immunohistochemistry of pulmonary IL-1β and TNF-α**

Lung sections were deparaffinized, rehydrated, and heated in sodium citrate buffer solution at pH 6.0 (Merck, Germany) using a microwave. Endogenous peroxidase activity was blocked in 3% H2O2 in distilled water. The sections were incubated with blocking buffer (normal goat serum) at room temperature for 30 min to block nonspecific binding sites. They were then incubated with an optimized primary antibody solution containing rabbit anti-mouse IL-1β and TNF-α antibodies (Abcam, Cambridge, MA, USA) in a humidified chamber at 4°C overnight before being incubated with appropriately diluted secondary antibodies in a humidified chamber at room temperature for 30 min. An avidin-biotin complex (VECTASTAIN ABC Kit, Vector Laboratories, USA) conjugated with horseradish peroxidase was added to the sections, and diaminobenzidine (DAB; Vector Laboratories, USA) was applied for 3 min. After counterstaining with Mayer’s hematoxylin (Merck, Germany), the sections were dehydrated and mounted. All slides were randomly scored in 50 microscopic fields at high magnification as follows: 0 = no immunopositive cells; 1 = 1–25% immunopositive cells; 2 = 26–50% immunopositive cells; 3 = 51–75% immunopositive cells; and 4 = >75% immunopositive cells [22].

**Statistical analysis**

The results are expressed as the mean ± standard error of the mean (SEM). Differences between groups were determined using one-way analysis of variance. Post hoc testing was performed for group comparisons using the least significant difference test. Values of p < 0.05 were considered to indicate significance.

**Results**

**Aqueous TL leaf extract contained total phenolic compounds and caffeic acid**

Based on the Folin-Ciocalteu colorimetric method, linear regression was performed between the GA concentrations and the absorbance (y = 0.0039x + 0.0414, “R2” = 0.9996). The phenolic content of the extract was 20.1 ± 0.39 μg GA Eq/mg extract. The linearity of HPLC-UV determination was in the concentration range of 1.56–100 μg/ml (“R2” = 0.9999). Within the range of analysis, the coefficient of variation was less than 3%. The caffeic acid content in the extract was 0.31 ± 0.01 μg/mg.

**Aqueous TL leaf extract alleviated PQ-induced increases in MDA levels in lung tissue**

As shown in Fig. 1, we evaluated the antioxidant effects of aqueous TL leaf extract against PQ-induced oxidative damage in male Wistar rats by detecting the levels of MDA, a biomarker of lipid peroxidation, using an assay kit. We found that the PQ group showed significantly higher MDA levels than the control group (p < 0.05). However, both the PQ + TL-LD group and the PQ + TL-HD group exhibited significantly lower MDA levels than the PQ group (p < 0.05).

**Aqueous TL leaf extract improved PQ-induced pathological alterations in lung tissue**

As shown in Figs. 2 and 3, the PQ group had significantly higher severity scores than the control group for pathological changes including alveolar edema, diffuse alveolar collapse, hemorrhage, leukocyte infiltration, alveolar septal thickening and vascular congestion (p < 0.001). The PQ + TL-LD group exhibited significantly less alveolar edema, diffuse alveolar collapse and hemorrhage than the PQ group (p < 0.05). Moreover, the PQ + TL-HD group demonstrated significantly less alveolar edema, diffuse alveolar collapse and hemorrhage, leukocyte infiltration, alveolar septal thickening and vascular congestion than the PQ group (p < 0.05) (Table 2).

**Aqueous TL leaf extract downregulated the expression of NOX, IL-1β and TNF-α in the lung tissues of PQ-treated rats**

As illustrated in Fig. 4, Supplementary File 1 and 2, we evaluated anti-inflammatory ability by detecting the

![Fig. 1 Pulmonary MDA levels of the study groups. The results are expressed as the means ± SEMs (n = 4 per group). *p < 0.05 compared with the control group. †p < 0.05 compared with the PQ group](image-url)
mRNA expression of NOX, IL-1β and TNF-α in the lung tissues of PQ-treated rats using RT-PCR. The results showed that the PQ group had significantly higher mRNA expression of NOX, IL-1β and TNF-α than the control group \((p < 0.05)\). However, both the PQ+TL-LD group and the PQ+TL-HD group exhibited significantly lower mRNA expression of IL-1β \((p < 0.001)\) than the PQ group, and the PQ+TL-HD group exhibited significantly lower mRNA expression of NOX \((p < 0.05)\) and TNF-α \((p < 0.05)\) than the PQ group.

**Aqueous TL leaf extract reduced the expression of IL-1β and TNF-α in the lung tissues of PQ-treated rats**

Immunohistochemistry showed that the PQ group had significantly higher expression of IL-1β and TNF-α than the control group \((p < 0.001\) and \(p < 0.05\), respectively). However, the PQ+TL-HD group exhibited significantly lower expression of IL-1β and TNF-α than the PQ group \((p < 0.05)\) (Fig. 5).

**Discussion**

PQ-induced lung pathology has been thoroughly examined in both humans and experimental animals. The severity of PQ poisoning has been reported to exhibit dose and time dependence \([23]\). This study demonstrated the occurrence of PQ toxicity in the lungs of experimental rats that were administered 18 mg/kg BW PQ by subcutaneous injection once a week for 6 weeks. The pathological changes included alveolar edema, diffuse alveolar collapse, hemorrhage, leukocyte infiltration, alveolar septal thickening and vascular congestion. The primary targets of PQ are type I and II pneumocytes and Clara cells, which accumulate PQ via the polyamine transport system \([24]\). In type I alveolar cells, PQ causes swelling followed by vacuolation and disruption of organelles \([4]\). In type II alveolar cells, PQ induces the apoptosis of human lung type II alveolar epithelial cells via the ER stress pathway in vitro \([25]\). Loss of type II pneumocytes affects the synthesis and secretion of surfactant, eventually resulting in increased intra-alveolar surface tension, alveolar edema and collapse \([26, 27]\). PQ poisoning also causes pulmonary hemorrhage as a result of increased capillary endothelial permeability and vasoconstriction of the respiratory bronchiolar arterioles \([3, 28]\).

Oxidative stress results from imbalance between the production and clearance of oxidants, including...
ROS and reactive nitrogen species, which can damage lipids, proteins and DNA [29]. Recently, studies on oxidative stress have widely used biomarkers such as MDA, an indicator of lipid peroxidation, to measure oxidative stress status [30]. Generally, the lipid peroxidation process comprises three steps, initiation, propagation and termination; during this process, oxidants attack lipids containing carbon-carbon double bonds, eventually producing lipid peroxidation products [31]. In this study, PQ treatment increased the levels of pulmonary MDA, indicating that the pathogenesis of PQ-induced lung damage is associated with ROS generation and oxidative stress [32]. ROS play a crucial role in oxidative stress upon PQ exposure, leading to the development of PQ-induced lung injury [6]. ROS are byproducts of aerobic metabolism and include mainly O$_2^-$, 'OH and H$_2$O$_2$. ROS are produced within cells and organelles, such as mitochondria,
peroxisomes, the endoplasmic reticulum and the plasma membrane [33]. \( \text{O}_2^- \), the most common ROS, is produced by transfer of an electron to molecular oxygen. Enzymes including xanthine oxidase, lipoxygenase, cyclooxygenase and NOX have been reported to be potential sources of \( \text{O}_2^- \) [33]. In this study, PQ increased the expression of NOX mRNA. The results indicated that PQ can induce ROS generation and oxidative stress in the lungs by upregulating NOX. Notably, PQ has been reported to stimulate leukocyte infiltration into the interstitial and alveolar spaces and to increase the production of pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1β [34]. This study demonstrated that PQ upregulated the mRNA expression of pro-inflammatory cytokines IL-1β and TNF-α, indicating that inflammation plays a crucial role in the mechanism by which PQ induces lung injury.

TL is a traditional Thai medicine belonging to the Acanthaceae family that is used as a therapeutic agent for alcohol and drug addiction [14]. Aqueous TL leaf extract has been reported to possess antioxidant, anti-diabetic, anti-inflammatory, and antipyretic properties [11]. The anti-oxidative stress effects of aqueous TL leaf extract have been shown both in vitro and in vivo [35, 36]. The results of this study showed that aqueous TL leaf extract reduced the mRNA expression of NOX and reduced the levels of pulmonary MDA in rats treated with PQ. We suggest that antioxidation via inhibition of NOX may be the key mechanism by which aqueous TL leaf extract attenuates PQ-induced oxidative stress in the lungs. In addition, in this study, aqueous TL leaf extract contained total phenolic compounds and caffeic acid, which have been shown to play key roles in antioxidant defense [37]. This extract has also been reported to have anti-inflammatory effects [38]. Moreover, our findings demonstrated that aqueous TL leaf extract downregulated IL-1β and TNF-α mRNA expressions in the lungs of rats treated with PQ, indicating that the extract induced anti-inflammatory effects to ameliorate PQ-induced lung injury. Surprisingly, this extract, especially at a high dose, attenuated pathological alterations in the lungs induced by PQ. We suggest that aqueous TL leaf extract can ameliorate PQ-induced lung pathology by alleviating oxidative damage and inflammation.

**Conclusion**

This study indicates that PQ causes oxidative stress and inflammation in male Wistar rats, leading to pathological alterations in the lungs. In addition, the results
reveal that aqueous TL leaf extract can ameliorate PQ-induced lung pathology by alleviating oxidative stress via inhibition of the expression of NOX and by attenuating inflammation via inhibition of the expression of the pro-inflammatory cytokines IL-1β and TNF-α. We propose that active compounds in this extract, particularly phenolic compounds and caffeic acid, may exert crucial protective effects against PQ-induced lung injury. Thus, this study suggests that aqueous TL leaf extract can be used as an antidote for PQ-induced lung injury.

Abbreviations
BW: Body weight; FW: Forward primer; H & E: Hematoxylin and eosin; H2O2: Hydrogen peroxide; HPLC: High-performance liquid chromatography; IL-1β: Interleukin 1 beta; MDA: Malondialdehyde; NOX: NADPH oxidase; O2•−: Superoxide radical; OH: Hydroxyl radical; PQ: Paraquat; ROS: Reactive oxygen species; RT-PCR: Reverse transcription-polymerase chain reaction; SEM: Standard error of the mean; TL: Thunbergia laurifolia; TNF-α: Tumor necrosis factor alpha.

Supplementary Information
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Authors’ contributions
SP designed this study and performed all experiments. CP, PK and PN carried out the immunohistochemistry. WP contributed to the mRNA expression analysis using RT-PCR. RK carried out the animal experiments and MDA level analysis. KB contributed to the HPLC analysis of the aqueous TL leaf extract. PC prepared the aqueous TL leaf extract. KB and GY analyzed the total phenolic and caffeic acid levels. PS carried out the pathological study. SP edited the final manuscript. All authors read and approved the final manuscript.

Fig. 5 Immunohistochemistry of pulmonary IL-1β and TNF-α in male Wistar rats (n = 4 per group). *p < 0.05 compared with the PQ group. **p < 0.05 compared with the control group.

Additional file 1.
Additional file 2.
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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information file.

Declarations

Ethics approval and consent to participate
All animal procedures were approved by the Animal Ethics Committee, Walailak University (Certification no. WU-AICUC-63-013).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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