Heliyon 8 (2022) e09234

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Thunbergia laurifolia aqueous leaf extract ameliorates paraquat-induced kidney injury by regulating NADPH oxidase in rats

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ARTICLE INFO

Keywords:
Paraquat
Thunbergia laurifolia
Oxidative stress
NADPH oxidase
Malondialdehyde

ABSTRACT

We aim to study the antioxidant ability of *Thunbergia laurifolia* (TL) aqueous leaf extract against PQ-induced kidney injury. Rats were divided into four groups (n = 4 per group): control group, the rats received subcutaneous injection of 1 ml/kg body weight (BW) normal saline; PQ group, the rats received subcutaneous injection of 18 mg/kg BW paraquat dichloride; PQ + TL-low dose (LD) group, the rats received subcutaneous injection of 18 mg/kg BW paraquat dichloride and were orally gavaged with TL leaf extract (100 mg/kg BW); and PQ + TL-high dose (HD) group, the rats received subcutaneous injection of 18 mg/kg BW paraquat dichloride and were orally gavaged with TL leaf extract (200 mg/kg BW). This study analyzed blood urea nitrogen (BUN) and creatinine levels, renal malondialdehyde (MDA) levels, kidney histopathology, mRNA expressions of renal NADPH oxidase (NOX) and protein expressions of renal NOX-1 and NOX-4 using immunohistochemistry. The PQ group showed a significant increase in BUN and creatinine levels, renal MDA level, and a upregulation of the mRNA expression of renal NOX compared with the control group. It also demonstrated mild hydropic degeneration of the tubules. Immunohistochemistry displayed a significant increase in the protein expressions of renal NOX-1 and NOX-4 compared with the control group. TL aqueous leaf extract especially in the high dose group significantly reduced the BUN and creatinine levels, the renal MDA level, and downregulated the mRNA expression of renal NOX and protein expressions of renal NOX-1 and NOX-4 compared with the PQ group. Furthermore, it can improve PQ-induced kidney injury. TL aqueous leaf extract can ameliorate PQ-induced kidney injury by regulating oxidative stress through inhibiting NOX, especially NOX-1 and NOX-4 expressions.

1. Introduction

Paraquat (PQ) is a highly toxic herbicide which is widely used in many countries of the world (Yu et al., 2014). Due to its severe toxicity and fatality rate of 60–80%, many countries have banned use of PQ (Kim et al., 2017; Weng et al., 2017). PQ toxicity causes severe acute and chronic health problems which may range from mild to fulminant and is commonly caused mortality (Safaei Asl and Dadashzadeh, 2016; Oa et al., 2013). Even with proper use, PQ ingestion can cause development of intracellular oxidative stress in multiple organs (Dinis-Oliveira et al., 2008). Previous studies have reported that PQ is a common cause of kidney injury in the patients (Weng et al., 2017; Isha et al., 2018). Histological examination of PQ causes mild hydropic degeneration of the proximal convoluted tubules in kidney (Lock and Ishmael, 1979). Oxidative stress is strongly implicated in the pathogenesis of kidney injury upon PQ exposure (Tan et al., 2015; Ranjbar et al., 2015). It occurred from the imbalance between reactive oxygen species (ROS) generation and antioxidant defenses (Liguori et al., 2018). ROS is mainly composed of superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), which are a highly reactive molecules to macromolecules leading to cellular and tissue damage (Schieber and Chandel, 2014). Nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase (NOX) family, a ROS-generating enzyme, plays a crucial role in PQ-induced oxidative stress (Cristóvão et al., 2009).

Due to lack of specific treatment, medicinal plants are investigated to explore specific and effective antidote against PQ poisoning (Suntres, 2018). *Thunbergia laurifolia* (TL) is commonly known as blue trumpet vine or laurel clock vine which is an important herb in Thai traditional medicine (Chan et al., 2011; Junsi et al., 2020). In Thailand, TL leaves are commonly consumed as herbal tea for detoxification purpose. It has been reported that they possess various pharmacological properties including antioxidant, anti-microbial, anti-proliferative, hepatoprotective, and

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https://doi.org/10.1016/j.heliyon.2022.e09234
Received 17 December 2021; Received in revised form 17 January 2022; Accepted 29 March 2022

anti-inflammatory with non-toxic effects (Chan et al., 2011). Therefore, this study aims to investigate whether TL aqueous leaf extract possesses beneficial effects to help alleviate PQ-induced kidney injury by inhibiting NOX.

2. Materials and methods

2.1. Herbal collection

The TL leaves were obtained from Nakhon Si Thammarat province, Thailand. Voucher specimen *Thunbergia laurifolia* AHS2008120101 was deposited at Herbarium of Plant Genetic Conservation Project under The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG), Walailak University, Nakhon Si Thammarat.

2.2. Extraction

The TL leaves were dried and ground into powder using a blender. The powder (10 g) was extracted with 100 ml of boiled distilled water. The filtrate was lyophilized in a freeze dryer (Eyela, Tokyo, Japan) (yield 13% w/w) and preserved at -80 °C for further investigation.

2.3. Animal treatments

All animal procedures were approved and performed in accordance with the Animal Ethics Committee, Walailak University (Certification no. no. 002/2018). This study was performed in a manner that minimized animal sufferings and the numbers of animals used in studies. Male Wistar rats (*Rattus norvegicus*) (n = 16) aged six weeks were obtained from Nomura Siam International Co, Ltd., Bangkok, Thailand. All of them were kept under controlled conditions of 23 ± 2 °C and 50–60% relative humidity with a 12 h light/dark cycle. They were provided access to diet and water. They were randomly divided into 4 groups: control group, the rats received subcutaneous injection of 1 ml/kg body weight (BW) normal saline once a week for 6 weeks; PQ group, the rats received subcutaneous injection of 18 mg/kg BW paraquat dichloride once a week for 6 weeks; PQ + TL-LD group, the rats received subcutaneous injection of 18 mg/kg BW paraquat dichloride once a week for 6 weeks and were orally gavaged with low dose TL leaf extract (100 mg/kg BW); and PQ + TL-HD group: the rats received subcutaneous injection of 18 mg/kg BW paraquat dichloride once a week for 6 weeks and were orally gavaged with high dose TL leaf extract (200 mg/kg BW) once a day for 6 weeks. PQ and TL treatments were performed according to the method of Orito et al. (2004), and Tangpong and Satarug (2010). The rats were euthanized with thiopental sodium overdose (100 mg/kg BW) anesthesia, then the kidneys were collected.

2.4. Biochemical analysis

Blood samples were centrifuged at 3000 rpm for 5 min. Sera were collected. Levels of BUN and creatinine were measured using Cobas Mira Plus CC Chemistry Analyzer (Switzerland).

2.5. Determination of MDA level

The measurement was performed using the OxiSelect™ TBARS Assay Kit (CAT no. STA-330, Cell Biolabs, Inc., USA) according to the manufacturer's protocol. The kidney tissues were cut into small pieces, washed by phosphate-buffered saline and homogenized to give a final concentration of 50 mg/mL in phosphate buffered saline (PBS) containing 1X butylated hydroxytoluene (BHT). The tissues were then homogenized on ice and centrifuged at 10000 × g for 5 min to collect supernatant. Then, 100 μL of samples or MDA standard was added to microcentrifuge tubes, 100 μL of the SDS lysis solution was added and mixed thoroughly. All the samples were incubated for 5 min at room temperature. Then, 250 μL of thiobarbituric acid (TBA) reagent was added. Each tube was closed and incubated at 95 °C for 60 min. The tubes were then removed and cooled to room temperature in an ice bath for 5 min. All tubes were then centrifuged at 3000 rpm for 15 min. The supernatant was removed and finally 200 μL of samples and MDA standard was transferred to a 96-well microplate compatible with a spectrophotometric plate reader. The absorbance was read at 532 nm.

2.6. Microscopic examination of histological alterations

The kidney section was fixed with 10% neutral buffered formalin solution, processed, and embedded in paraffin. It was sliced into 5 μm section using a microtome and then stained with hematoxylin and eosin (H&E). The sample was observed under light microscope (Olympus BX53F2, Japan).

2.7. Determination of mRNA expressions of renal NOX using reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a Tissue Total RNA Mini Kit (Geneaid, Korea). The purity and quantity of RNA were analyzed using NanoDrop™ one/one^{C} Microvolume UV-Vis Spectrophotometer with Wi-Fi (Thermo Scientific™, USA). Reverse transcription and PCR were performed for amplification. The thermal cycling conditions were set up as follows: denaturation at 95 °C for 15 min and at 94 °C for 1 min, annealation at 65 °C for 1 min, extension at 72 °C for 1 min, and elongation at 72 °C for 10 min. The primers contained NOX: forward primer, GGAAATA-GAAAGTTGACTGGCCC and reverse primer, GTATGAGTGCCATCCAGAG

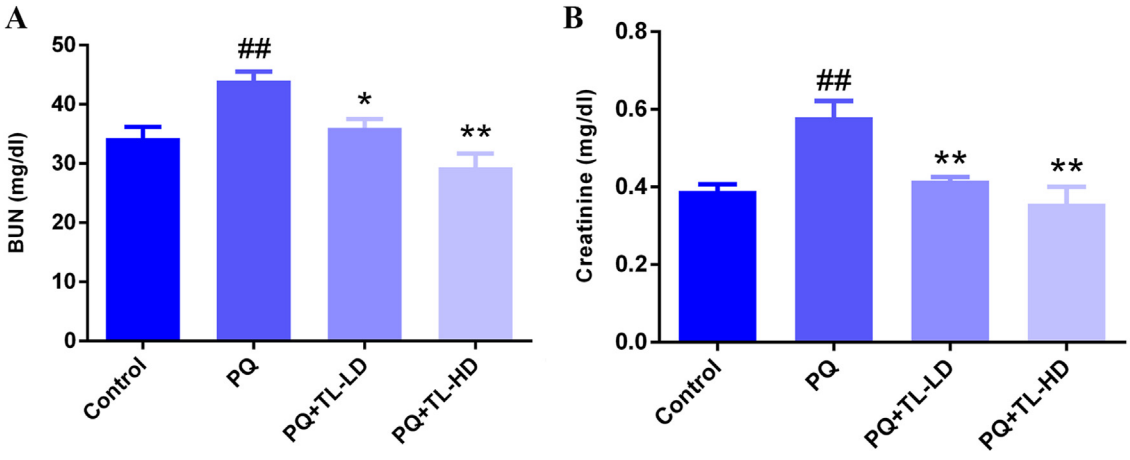

Figure 1. Effect of TL aqueous leaf extract on BUN (A) and creatinine (B) levels. The results are expressed as mean ± SEM (n = 4 per group). $^{\#\#}p < 0.001$ compared with the control group. $^{*}p < 0.05$, $^{**}p < 0.001$ compared with the PQ group.

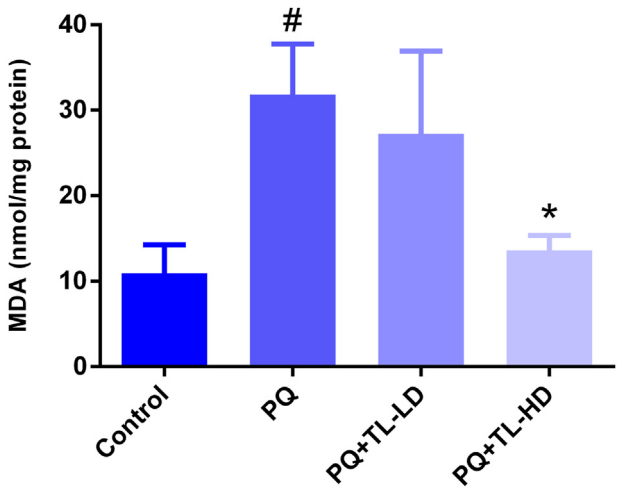

Figure 2. Renal MDA levels of study groups. The results are expressed as mean ± SEM (n = 4 per group). $^{\#}p < 0.05$ compared with the control group. $^{*}p < 0.05$ compared with the PQ group.

CAG (Rashed et al., 2011); and β-actin: forward primer, TTCTTTGCAGCTCCTTCGTTGCCG and reverse primer, TGGATGGCTACGTACATGGCTGGG (Bessa et al., 2012). The primer sequence was 5′-3′. The sample was examined on 2 % gel agarose. Following ethidium bromide staining, the bands were visualized using an UV trans-illuminator. The density of PCR product was analyzed using GeneTools image analysis software (Syngene, Frederick, MD, USA).

2.8. Immunohistochemistry of the renal NOX-1 and NOX-4

After deparaffinization, the section was rehydrated and heated in sodium citrate buffer solution at pH 6.0 (Merck, Germany). Then the slide was incubated with 3% H_2O_2 in distilled water for blocking endogenous peroxidase activity. The blocking buffer (normal goat serum) was used for blocking nonspecific binding site. The slide was incubated with a primary antibody containing rabbit anti-mouse NOX-1 (Santa Cruz Biotechnology Inc., USA) and NOX-4 (Santa Cruz Biotechnology Inc., USA) overnight, then incubated with secondary antibody (Vector Laboratories, CA, USA). Avidin-biotin complex (Vectastain ABC Kit, Vector Laboratories, USA) conjugated with horseradish peroxidase was added to the sections and the DAB Kit (Vector Laboratories, USA) was applied. The sections were then counterstained with Mayer's hematoxylin (Merck, Germany), and were dehydrated and mounted. The slide was scored in 50 random microscopic fields at high magnification: score 0 = none; score 1 = 1–25% of immunopositive cell; score 2 = 26–50% of immunopositive cell; score 3 = 51–75% of immunopositive cell; and score 4 > 75% of immunopositive cell.

2.9. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences between the groups were determined using one-way analysis

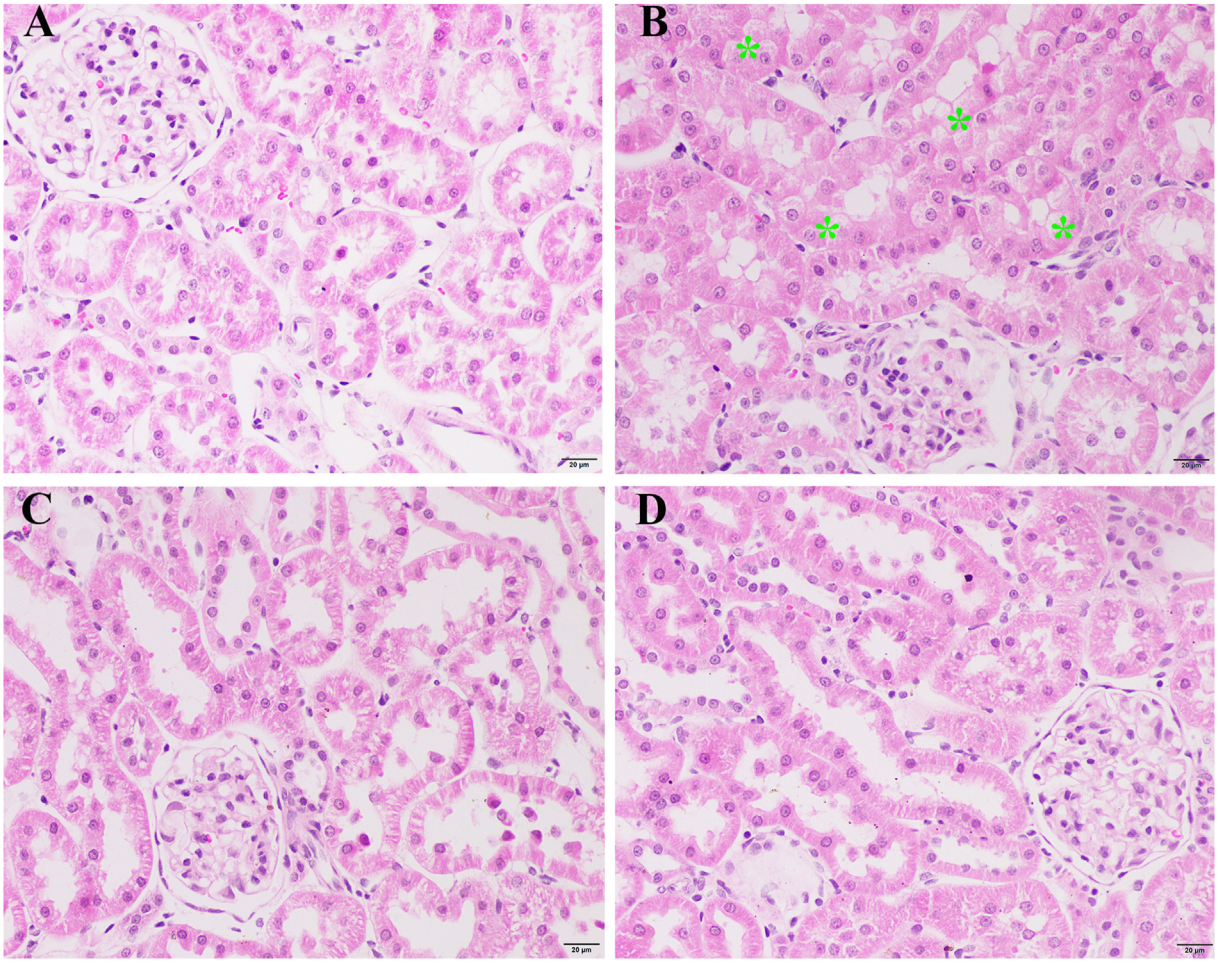

Figure 3. Kidney microscopic examinations of male Wistar rats (H&E staining) of the control (A), the PQ (B), the PQ + TL-LD (C) and the PQ + TL-HD (D) groups. The green asterisk indicates hydropic degeneration of the tubules. Scale bar = 20 μm.

of variance followed by the least significant difference test (LSD). P values of <0.05 were considered statistical significance.

2.10. Ethical statement

All animal procedures were carried out in accordance with the guidelines of the Animal Ethics Committee of Walailak University (certification no. 002/2018).

3. Results

3.1. Effects of TL aqueous leaf extract on BUN and creatinine in PQ-treated rats

As illustrated in Figure 1, the PQ group showed a significant increase in the levels of BUN and creatinine ($p < 0.001$) compared with the control group. The PQ + TL-LD group demonstrated a significant decrease in the levels of BUN ($p < 0.05$) and creatinine ($p < 0.001$) compared with the PQ group. Moreover, The PQ + TL-HD group also demonstrated a significant decrease in the levels of BUN ($p < 0.05$) and creatinine ($p < 0.001$) compared with the PQ group.

3.2. Effects of TL aqueous leaf extract on renal MDA levels in PQ-treated rats

The PQ group showed a significant increase in the renal MDA levels compared with the control group ($p < 0.05$). TL aqueous leaf extract in the low dose group had no statistically significant difference of the renal MDA levels compared with the PQ group. Fortunately, the high dose of TL aqueous leaf extract significantly decreased the renal MDA levels compared with the PQ group ($p < 0.05$) (Figure 2).

3.3. Effects of TL aqueous leaf extract on pathological alterations of kidney in PQ-treated rats

The PQ group showed mild hydropic degeneration of the tubules. TL aqueous leaf extract both low dose and high dose can improve the pathological alterations of the kidney (Figure 3).

3.4. Effects of TL aqueous leaf extract on the renal NOX expression in PQ-treated rats

The PQ group demonstrated a significant upregulation in the mRNA expression of renal NOX compared with the control group ($p < 0.001$). However, TL aqueous leaf extract of both low and high dose significantly downregulated the mRNA expression of renal NOX compared with the PQ group ($p < 0.001$) (Figure 4, Supplementary data 1).

3.5. Effects of TL aqueous leaf extract on immunohistochemistry of renal NOX-1 and NOX-4 in PQ-treated rats

The PQ group showed a significant increase in the expressions of renal NOX-1 ($p < 0.05$) and NOX-4 ($p < 0.001$) compared with the control group. Fortunately, TL aqueous leaf extract of both low and high dose significantly reduced the expressions of renal NOX-1 and NOX-4 ($p < 0.05$) compared with the PQ group (Figure 5).

4. Discussion

PQ is considered a highly toxic herbicide in the world. PQ poisoning in human primarily occurs due to accidental or intentional ingestion which caused fatal multiple organ failure mediated by ROS (Kim et al., 2008). This study demonstrated that PQ caused elevated BUN and serum creatinine levels similar to previous work (Kan et al., 2012). BUN and creatinine are nitrogenous end products of metabolism which are most widely used as a valuable screening test to evaluate kidney damage (Nisha and Srinivasa Kannan, 2017). BUN is a non-protein nitrogenous end product. The concentration of BUN depends on protein intake, body's capacity to catabolize protein, and capacity to excrete BUN by the renal system (Salazar, 2014). Creatinine is also a non-protein nitrogenous waste product generated by the breakdown of creatine and phosphocreatine which is accepted as an indicator to evaluate renal function (Price and Finney, 2000). Previous study demonstrated that PQ poisoning caused a markedly increase in creatinine levels, which is associated with loss of renal function indicated by increased generation of creatine and creatinine following severe oxidative stress (Mohamed et al., 2015). This study indicated that elevated levels of BUN and serum creatinine are associated with PQ-induced kidney injury.

Lipid peroxidation is involved in PQ poisoning resulting from the induction of ROS, eventually causing pathological alterations of cells and tissues (Bus et al., 1976). MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells and is used as a lipid peroxidation biomarker. Overproduction of MDA indicates oxidative damage (Gaweł et al., 2004). This study demonstrated that PQ caused an increase in the level of renal MDA similar to previous study (Amanov et al., 1994). The level of plasma MDA also increased in PQ intoxication (Yasaka et al., 1981). This study demonstrated that an increase in the level of renal MDA indicated oxidative damage and might reflect ROS generation in PQ-treated rats. NOX family is an important source of ROS production in biological system. It can be characterized into seven isoforms including NOX-1, 2, 3, 4 and 5, and DUOX-1 and 2 (Burtenshaw et al., 2017). It is a membrane-bound enzyme complex that transfers electrons across biological membranes and is involved in the pathogenesis of oxidative stress (Bedard and Krause, 2007). This study showed that mRNA expression of renal NOX upregulated in the PQ-treated rats.

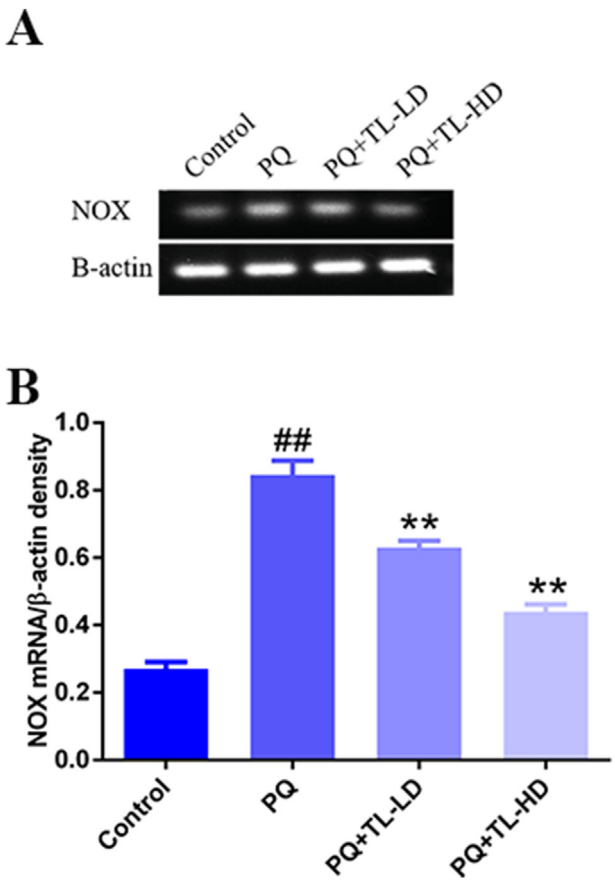

Figure 4. The mRNA expressions (A) and the mRNA density of renal NOX (B) of the study groups. Uncropped gel image was shown in Supplement data 1. The results are expressed as mean ± SEM (n = 4 per group). $^{\#\#}p < 0.001$ compared with the control group. $^{**}p < 0.001$ compared with the PQ group.

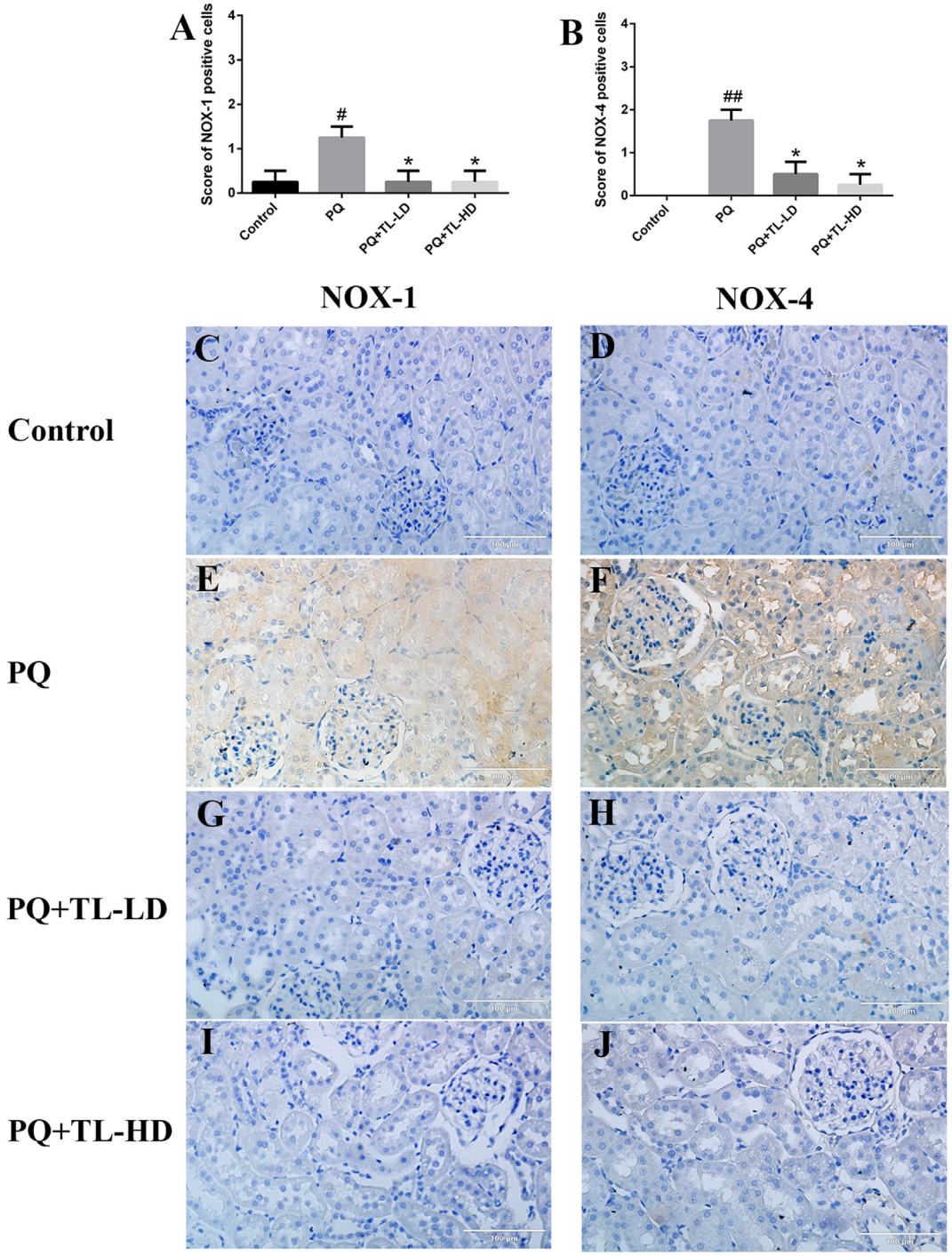

Figure 5. The immunohistochemistry of renal NOX-1 and NOX-4. The scores of renal NOX-1 (A) and NOX-4 (B) of the study groups. The expressions of renal NOX-1 and NOX-4 of the control (C, D), the PQ (E, F), the PQ + TL-LD (G, H) and the PQ + TL-HD (I, J) groups, respectively. Results are expressed as mean ± SEM (n = 4 per group). $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ compared with the control group. $^{*}p < 0.05$ compared with the PQ group. Scale bar = 100 μm.

Furthermore, immunohistochemistry illustrated the increased protein expressions of renal NOX-1 and NOX-4. We suggested that NOX especially NOX-1 and NOX-4 is implicated in the pathogenesis of oxidative stress following PQ exposure.

Currently, there is no specific effective antidote for treating PQ poisoning. TL is one of the most important Thai herbs and is commonly used in Thai traditional medicine. In Thailand, herbal tea from TL leaf is widely known for detoxification. Moreover, aqueous leaf extract of TL is found to exhibit low toxicity in prolonged use and serve as a significant anti-mutagenic activity (Saenphet et al., 2005; Chivapat et al., 2010). It also possesses various beneficial properties such as antioxidant, anti-inflammation and anti-microbial (Junsi et al., 2020; Wonkchalee et al., 2012; Chan et al., 2011). We hypothesized that TL aqueous leaf extract might possess antioxidant properties that help alleviate PQ-induced kidney injury by inhibiting NOX. This study found that both low dose and high dose of TL aqueous leaf extract could reduce the levels of BUN and creatinine, downregulate the mRNA expression of renal NOX, reduce protein expressions of renal NOX-1 and NOX-4, and alleviate kidney injury following PQ treatment, possibly due to its antioxidant abilities. The result showed that only a high dose of TL aqueous leaf extract could reduce the level of MDA. The modulating properties might result from the potential role of its aqueous extract constituents. Previous

study demonstrated that aqueous TL leaf extract contained primarily caffeic acid and apigenin using high-performance liquid chromatography (Oonsivilai et al., 2007). Taken together, we found that TL aqueous leaf extract possessed antioxidant properties, which could modulate PQ-induced kidney injury by regulating oxidative stress through inhibiting NOX, especially NOX-1 and NOX-4, expressions.

5. Conclusions

PQ caused an increase in the levels of BUN, serum creatinine and renal MDA. Additionally, it induced upregulated mRNA expression of renal NOX. Immunohistochemistry demonstrated an increase in renal NOX-1 and NOX-4 expressions. This study shows that a high dose of TL aqueous leaf extract is more effective than a low dose in that it possesses antioxidant properties, which help reduce renal MDA level, downregulate mRNA expression of renal NOX and reduce protein expressions of renal NOX, specifically NOX-1 and NOX-4. As a result, it can alleviate kidney pathology induced by PQ.

Declarations

Author contribution statement

Sarawoot Palipoch: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Chuchard Punsawad: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Phanit Koomhin: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Wasinee Poonsawat: Performed the experiments.

Funding statement

This work was supported by a grant from the Institute of Research and Development (under the contract WU 61111) and by the new strategic research project (P2P) Walailak University, Thailand.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e09234.

Acknowledgements

We are thankful to the President and all staff of Walailak University for their kind support.

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