

## ***Morus alba* L. Leaf Extract Exerts Anti-Inflammatory Effect on Paraquat-Exposed Macrophages**

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### **Abstract**

Paraquat (PQ) is a widely used herbicide in agricultural areas. However, it has been reported to cause harm to animals or humans exposed to this herbicide. Generally, PQ stimulates the production of reactive oxygen species within cells causing cell death and resulting in bodily abnormalities. At present, efforts are being made to find drugs to reduce the toxic effects of PQ and natural products are targets of interest. This study aimed to assess the effect of mulberry (*Morus alba* L.) leaf extract on macrophages (RAW 264.7) after exposure to PQ, including the viability and expressions of NF- $\kappa$ B, IL-1 $\beta$ , and IL-6. We treated RAW 264.7 macrophages with *Morus alba* L. leaf extract in the absence or presence of PQ. Subsequently, viability, apoptosis, and expressions of NF- $\kappa$ B, IL-1 $\beta$ , and IL-6 were examined. It was found that the extract of mulberry leaves could inhibit apoptosis, increase viability, and significantly downregulates expressions of NF- $\kappa$ B, IL-1 $\beta$ , and IL-6 when RAW 264.7 macrophages were exposed to PQ. These phenomena indicated that *Morus alba* L. leaf extract has properties to reduce the inflammatory effect caused by the herbicide PQ.

**Keywords:** Paraquat, *Morus alba* L. leaf extract, RAW 264.7 macrophages, NF- $\kappa$ B, IL-1 $\beta$ , IL-6

### **Introduction**

The widespread use of herbicides in the agriculture sector for control of weeds and grass in plantation crops is an important concern throughout the globe [1]. These herbicides contaminate the environment, especially animal feed, and roughage. As a result, they may accumulate in animals and humans through direct or indirect contact with the residue, thereby constituting a health risk.

Paraquat (PQ) is a highly toxic and hazardous herbicide that is widely used in both agricultural and non-agricultural areas [2]. It was reported that PQ exerts several toxic effects on different organs such as the lung, kidney, and immune system and subsequently results in death [3,4]. Toxicity of PQ relates to generating reactive oxygen species (ROS) [4]. Excess production of ROS by PQ-induced oxidative stress damages cellular structure and cell function, causing many pathological effects [1]. In addition, PQ causes cytotoxicity, for example, lipid peroxidation, and damage to mitochondria DNA [1]. Furthermore, overproduction of PQ-induced ROS promotes the activity of numerous cytokines including interleukin-1beta (IL-1 $\beta$ ), IL-6, IL-18, and tumor necrosis factor-alpha (TNF- $\alpha$ ), and cell apoptosis [3,5,6]. PQ enhances inflammatory response by suppressing anti-inflammatory cytokines [3]. Moreover, ROS can activate nuclear factor kappa B (NF- $\kappa$ B) which regulates inflammatory response and cell survival [6].

Currently, there are no effective treatment methods for poisoning by PQ. Therefore, development of novel therapeutic agents for PQ that contain antioxidant or anti-inflammatory activities is of importance. At present, studies are being attempted to develop potential natural plant products for use in immune modulation and treatment of poisoning caused by PQ toxicity.

Mulberry (*Morus alba* L.) is classified in the family Moraceae. Mulberry leaves are traditional herbs used for antipyretic, cough suppressants, anti-hyperglycemia, and anti-hypertension [7]. It has been used in herbal medicine in many parts of the world because the mulberry tree contains multiple chemical components with biological activities, for example, flavonoids, phenolic acids, and alkaloids [8]. These natural bioactive compounds have various biological properties and potent pharmacological benefits including anticancer [8], antioxidation [9], and anti-inflammation [10]. However, little is known about using *Morus alba* L. leaf (ML) extract for the treatment of PQ toxicity or of the molecular mechanisms of the extract from this plant.

Thus, this research aimed to determine the anti-apoptosis and anti-inflammatory activity of ML extract on PQ-exposure macrophages. Knowledge from these studies may provide useful information to develop natural antioxidant and anti-inflammatory drugs for reducing the toxicity of PQ.

## Materials and methods

### Preparing extracts from *Morus alba* L. leaves

Fresh mulberry leaves were harvested in July 2021 at Mahasarakham University. Leaves were oven-dried at 60 °C for 6 h and then powdered. Powdered leaves were extracted using 95 % ethanol, then filtered, and freeze-dried. The extract was dissolved in dimethyl sulfoxide (DMSO). Then, the mixture was filtered through a 0.22-µm membrane filter (Minisart, Germany) and kept at -20 °C for subsequent use.

### Cell culture

RAW 264.7 macrophages were used throughout this study. Cells were maintained in DMEM (HyClone, USA) with 10 % FBS (Sigma-Aldrich, USA), and 1 % antibiotic (penicillin-streptomycin, Gibco) at 37 °C in 5 % CO<sub>2</sub>.

### *In vitro* cytotoxic test by MTT assay

Cytotoxicity of ML extract and PQ to macrophages was measured by MTT assay. Briefly, RAW 264.7 macrophages were seeded overnight in 100 µL medium/well of a 96-well plate (1.5×10<sup>4</sup> cells/well). Then the cells were treated with 0 - 1.4 mM of PQ and 0 - 30 µg/mL of ML extract for 24 and 48 h, and DMSO (AppliChem, Germany) was used as a vehicle. Finally, MTT (5 mg/mL, Abcam) reagent was added and cultured at 37 °C for 4 h. After the medium was removed, formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm by a microplate reader (TECAN, Switzerland).

### Detection of cell apoptosis by acridine orange-ethidium bromide (AO-EB) staining

Cell apoptosis was determined using AO/EB fluorescence staining based on the method described previously [11]. Briefly, cell suspensions of 6,000 cells were cultured per well of a 96-well plate overnight, then incubated with various concentrations of ML extract for 24 h before exposure with PQ. After 48 h incubation, each well was stained with dual AO and EB dyes (1:1 ratio) for 5 min. After staining, the percentages of apoptotic cells were counted immediately under a fluorescence microscope (Nikon, Japan).

### Quantitative RT-PCR analysis

Total RNA was extracted and converted to cDNA using the Nucleospin RNA kit (Macherey-Nagel, Germany) and ReverTra Ace qPCR RT Kit (TOYOBO, Japan) respectively. The relative gene expression was subsequently amplified using a QuantStudio™3 Real-Time PCR (Applied Biosystems). For each qPCR reaction reagent was mixed using Thunderbird SYBR qPCR Mix (TOYOBO, Japan). The gene expression levels were analyzed by normalizing to GAPDH. The primer sequences are described in **Table 1**.

**Table 1** The sequences of primers.

Gene	Primer sequences (5' to 3')	References
<i>IL-6</i>	F: TTCCATCCAGTTGCCTTCTTG	[12]
	R: CATTTCACGATTTCCAGAG	
<i>IL-1β</i>	F: GACGGACCCAAAAGATGAAG	[12]
	R: CTCCACAGCCACAATGAGTGA	
<i>NF-κB p65</i>	F: GCTCCTAAGGTGCTGACA	[13]
	R: ACCTCCGAAAGCGAGATA	
GAPDH	F: GGCATTGTGGAAGGGCTCAT	[12]
	R: GACACATTGGGGGTAGGAACAC	

### Statistical analysis

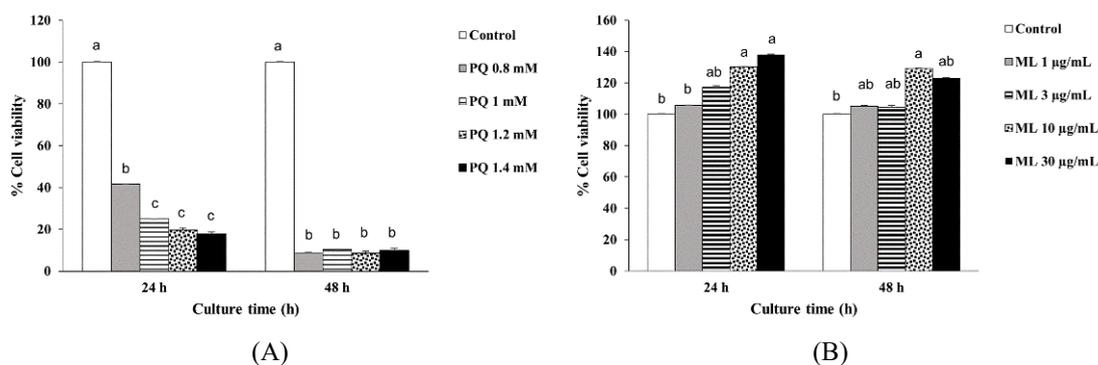
Differences between treatments were performed using one-way ANOVA followed by Duncan's multiple tests.  $p < 0.05$  was considered statistically significant.

### Results and discussion

#### Effect of PQ and ML extract on viability of RAW 264.7 macrophages

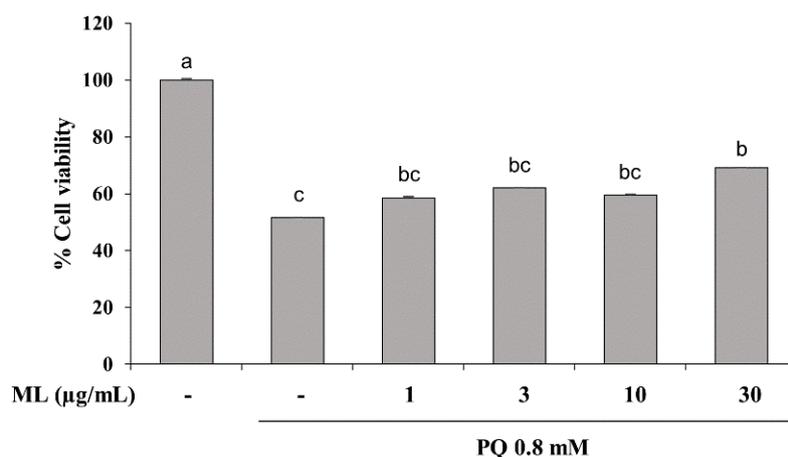
Effects of PQ and ML extract on cell viability were evaluated on RAW 264.7 macrophages using an MTT assay. To observe the effect of PQ on cell viability, macrophages were cultured in different concentrations of PQ. As shown in **Figure 1(A)**, after 24 h of exposure to PQ, the viability of macrophages incubated in PQ at 1, 1.2 and 1.4 mM was lower than the control group and the group incubated in PQ at a concentration of 0.8 mM ( $p < 0.05$ ). After 48 h of exposure to PQ, the viability of macrophages incubated in PQ at all concentrations was significantly lower than the control group ( $p < 0.05$ ). These results suggested that PQ has a direct effect on the viability of macrophages. Effects are also in expose-time and PQ-concentration dependent manners. The results further confirm our previous study that PQ increased the cytotoxicity of bone marrow-derived macrophages [14] and the previous report that PQ decreased human neural progenitor cells [15].

We further observed effect of ML extract on cell viability, macrophages were cultured in different concentrations of ML extract. As shown in **Figure 1(B)**, at 24 h, viability of macrophages incubated in ML extract at 10 and 30  $\mu\text{g}/\text{mL}$  was significantly higher than that of the control group and the group that incubated in ML extract at 1  $\mu\text{g}/\text{mL}$  ( $p < 0.05$ ). At 48 h, the viability of macrophages incubated in ML extract at a concentration of 10  $\mu\text{g}/\text{mL}$  was significantly higher than that of the control group ( $p < 0.05$ ). The result of this study was consistent with previous report, which described the phenomenon as ML extract enhanced  $\beta$ -cell proliferation and rescued  $\beta$ -cell death by diminishing endoplasmic reticulum stress [16]. A previous study demonstrated that polysaccharides from mulberry fruit could stimulate proliferation of splenocytes [9].



**Figure 1** Effect of PQ (A) and ML extracts (B) on viability of RAW 264.7 macrophages at 24 and 48 h. The values with different letters are significantly different ( $p < 0.05$ ).

Then we studied cytoprotective effect of ML extract on PQ-treated cells, macrophages were pre-treated with different concentrations of ML extract for 24 h and then exposed to PQ at a concentration of 0.8 mM for 24 h. As shown in **Figure 2**, viability of macrophages after pre-treated with ML extract at a concentration of 30  $\mu\text{g}/\text{mL}$  was significantly higher than the group exposed to PQ without pre-treatment ( $p < 0.05$ ). This finding was consistent with other studies on the effect of ML extracts on reducing toxicity of some substances, such as reduction of acetaldehyde toxicity from the alcohol-related liver disease [17] or reduction of toxicity of paracetamol on rat hepatocytes [18].

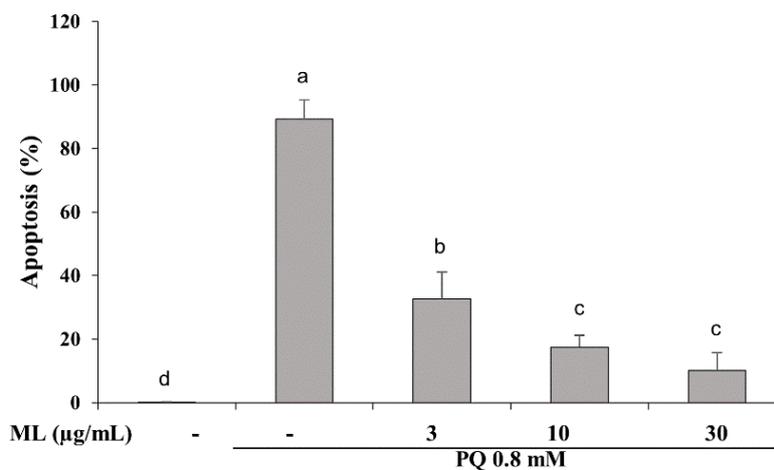


**Figure 2** Effect of ML extracts on viability of RAW 264.7 macrophages after exposure to PQ. Values with different superscripts differ significantly ( $p < 0.05$ ).

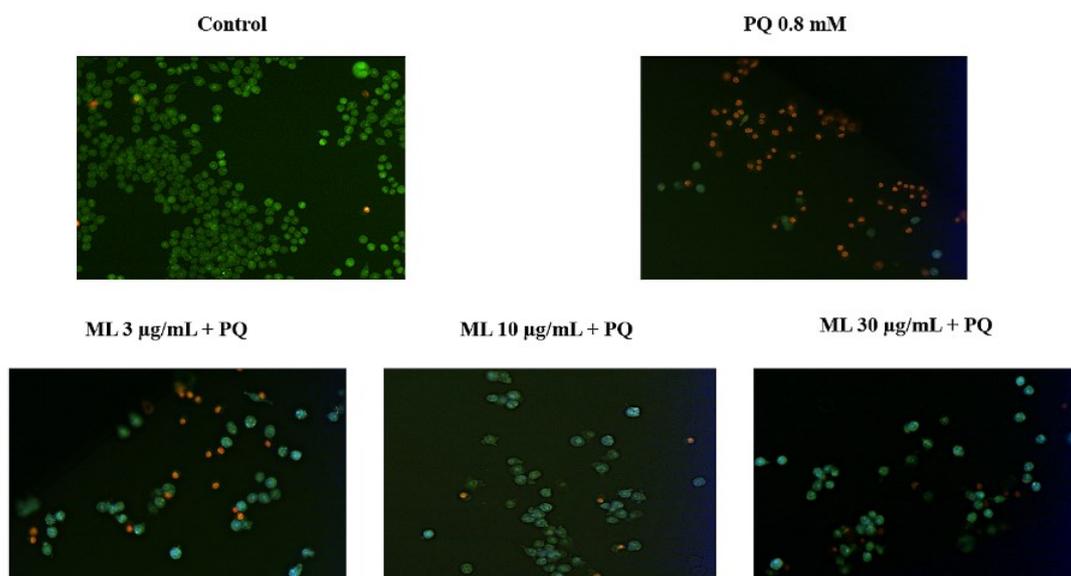
#### Effect of PQ on apoptosis of RAW 264.7 macrophages after pre-treated with mulberry leaves extract

After pre-treatment with ML extract, cells were stimulated with 0.8 mM PQ for 24 h. As shown in **Figure 3**, the percentage of apoptotic RAW 264.7 macrophages that had been pre-treated with the extract at a concentration of 10 and 30  $\mu\text{g/mL}$  was significantly lower than those pre-treated with the extract at concentrations of 3  $\mu\text{g/mL}$  ( $p < 0.05$ ). In addition, the percentage of apoptosis in all groups which had been pre-treated with the extract was significantly lower than in the group exposed to PQ without pre-treatment ( $p < 0.05$ ). Further, morphological and color changes of RAW 264.7 macrophages were verified by AO/EB staining. Green and orange to red color nuclei represent the live cell and apoptotic cells, respectively (**Figure 4**).

Apoptosis constitutes one of the major mechanisms underlying pathological characterized caused by PQ [5]. We have previously shown that PQ caused apoptosis in macrophages [14]. To further elucidate the effects of ML extracts in PQ-induced apoptosis, thus cell apoptosis was subsequently performed. We found that apoptosis was lower than that of the group exposed to the PQ without pre-treated with the extract. Others showed that ML ameliorated apoptosis in autoimmune myocarditis [19]. In addition, ML extract suppressed apoptosis in alcohol-induced hepatocytes [17]. This phenomenon showed that ML extract could reduce apoptosis and increase the viability of macrophages which observations support each other.



**Figure 3** Apoptosis percentage of RAW 264.7 macrophages. Cells were pre-treated with ML extracts for 24 h before exposing to PQ for 24 h. The values with different letters are significantly different ( $p < 0.05$ ).



**Figure 4** Characteristic and color of live and apoptotic RAW 264.7 macrophages were pre-treated with ML extracts for 24 h before exposing to PQ for 24 h.

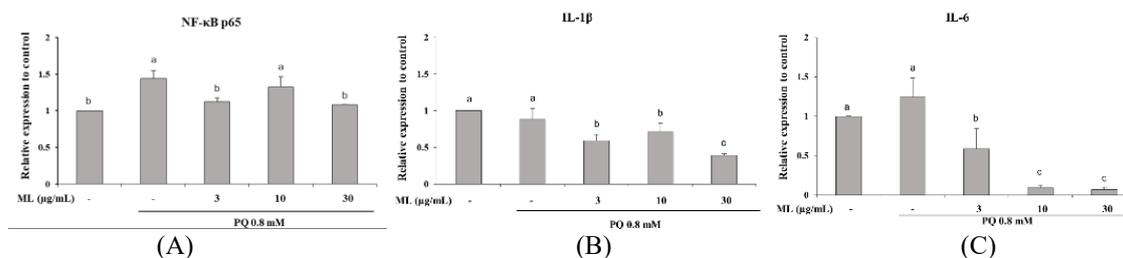
#### Effect of PQ on NF- $\kappa$ B, IL-1 $\beta$ and IL-6 gene expression of RAW 264.7 macrophages after pre-treatment with ML extract

Cells were incubated with ML extract for 24 h and then exposed to PQ at a concentration of 0.8 mM for 24 h. In our study, NF- $\kappa$ B p65 gene expression of the groups that had been pre-treated with the extract at 3 and 30  $\mu$ g/mL was significantly lower than that of the group that had been exposed to PQ without pre-treatment ( $p < 0.05$ ). However, NF- $\kappa$ B p65 gene expression of the group that pre-treated with the extract at 10  $\mu$ g/mL was not significantly different from the group that was exposed to PQ without pre-treatment ( $p > 0.05$ ) (**Figure 5(A)**). Moreover, IL-1 $\beta$  gene expression of the groups that had been pre-treated with the extract at 30  $\mu$ g/mL was significantly lower than that of the group pre-treated with the extract at 3 and 10  $\mu$ g/mL ( $p < 0.05$ ). In addition, IL-1 $\beta$  gene expression of the group that had been pre-treated with the extract at 3 and 10  $\mu$ g/mL was significantly lower than the group that was exposed to PQ without pre-treatment ( $p < 0.05$ ) (**Figure 5(B)**). We further evaluated the gene expression of IL-6 and it was found that IL-6 gene expression of the groups that had been pre-treated with the extract at 10 and 30  $\mu$ g/mL was significantly lower than that of the group pre-treated with the extract at concentrations of 3  $\mu$ g/mL ( $p < 0.05$ ). In addition, IL-6 gene expression of the group that had been pre-treated with the extract at a concentration of 3  $\mu$ g/mL was significantly lower than the group that was exposed to PQ without pre-treatment ( $p < 0.05$ ) (**Figure 5(C)**).

NF- $\kappa$ B has an essential function in inflammatory responses and is related to several inflammatory genes of macrophages [20]. Inhibition of the PQ-induced signalling pathway has been proposed as a target of novel therapeutic strategies resulting from this kind of poison. In our study, we found that the extract at 3 and 30  $\mu$ g/mL caused decreased NF- $\kappa$ B p65 gene expression which was lower than in the group that was exposed to PQ without pre-treated with the extract. Our findings corroborate the results of previous studies, which found that mulberry leaves attenuated TNF- $\alpha$  and LPS-induced lectin-like oxidized LDL receptor-1 mRNA expression by suppressing the NF- $\kappa$ B pathway [21]. In addition, a previous study reported that Kuwanon X from mulberry leaves acted against the herpes simplex virus type 1-induced NF- $\kappa$ B activation via inhibition of nuclear translocation [18]. Therefore, the result from present study confirms that ML extract could suppress the effect of PQ through NF- $\kappa$ B signalling inhibition in RAW 264.7 macrophages.

Pro-inflammation cytokines are associated with the inflammatory response [20]. Here, we found that the macrophages had lower IL-1 $\beta$  gene expression than that of the group exposed to PQ without pre-treatment with the extract. Besides, the extract at 30  $\mu$ g/mL had a lower IL-1 $\beta$  gene expression than the extract at other concentrations. This result was in agreement with the report, which found that ML extract was effective in its anti-inflammatory activities via inhibition of the expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  [10].

Additionally, IL-6 gene expression of RAW 264.7 macrophages pre-treated with ML extract at concentrations of 3, 10 and 30  $\mu\text{g/mL}$  had lower IL-6 gene expression levels than that of the group that was exposed to PQ without pre-treatment with the extract. Leaf extract at 10 and 30  $\mu\text{g/mL}$  had a level of IL-6 gene expression lower than that of the extract at 3  $\mu\text{g/mL}$ . This phenomenon was consistent with the report, which found that ML extract could inhibit a signal pathway that was promoted by IL-6 and TNF- $\alpha$  derived from adipocytes [22]. Moreover, previous study suggested that neochlorogenic acid which was extracted from mulberry leaves could reduce the expression of inflammatory-related proteins [23].



**Figure 5** Effect of PQ on NF- $\kappa$ Bp65 (A), IL-1 $\beta$  (B), and IL-6 (C) gene expression of RAW 264.7 macrophages after pre-treated with ML extract. Values with different superscripts differ significantly ( $p < 0.05$ ).

## Conclusions

This study evaluated the protective effect of ML extract on PQ-treated RAW 264.7 macrophages. The results showed that the leaf extract alleviated the toxicity of PQ by decreasing apoptotic cells of RAW 264.7 macrophages. In addition, the extract could attenuate an inflammatory response caused by PQ in RAW 264.7 macrophages through suppression of NF- $\kappa$ B, IL-1 $\beta$ , and IL-6. Therefore, these findings may provide novel knowledge for developing a natural product as a drug for the therapy of PQ-caused cytotoxicity.

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