



Protective effects of *Morus alba* L. leaf polyphenols against paraquat-induced apoptosis in macrophages

[Efectos protectores de los polifenoles de las hojas de *Morus alba* L. contra la apoptosis inducida por paraquat en macrófagos]

Jaroon Wandee^{1,2}, Piyarat Srinontong^{1,2*}, Worapol Aengwanich^{1,3}, Wilasinee Srisanyong⁴

¹Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, 44000, Thailand.

²Biovetterinary Research Unit, Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, 44000, Thailand.

³Stress and Oxidative Stress in Animal Research Unit, Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, 44000, Thailand.

⁴Department of Veterinary Technology, Faculty of Agriculture Technology, Kalasin University, Kalasin 46000, Thailand.

*E-mail: piyarat@msu.ac.th

Abstract

Context: Paraquat (PQ) induces cytotoxicity primarily through the generation of intracellular reactive oxygen species (ROS), leading to apoptosis. *Morus alba* L. leaves are rich in polyphenols and known for their potent antioxidant properties.

Aims: To quantify the total phenolic content, antioxidant capacity, and antioxidant activity of polyphenol extracts from *Morus alba* L. leaves (PEFML) and evaluate their protective effect against PQ-induced cytotoxicity in RAW264.7 macrophages.

Methods: Total phenolic content, total antioxidant capacity, and antioxidant activity of PEFML were assessed using standard *in vitro* assays. RAW264.7 macrophages were pretreated with PEFML for 24 hours prior to exposure to PQ (0.8 mM) for an additional 24 hours. Subsequently, cell viability, apoptosis, intracellular ROS generation, and the Bax/Bcl-2 mRNA expression ratio were analyzed.

Results: PEFML demonstrated significant antioxidant activity, as indicated by high phenolic content and strong antioxidant capacity. PEFML at concentrations ranging from 1.2 to 9.6 µg/mL exhibited no cytotoxicity in RAW264.7 cells. Notably, PEFML significantly suppressed intracellular ROS generation, particularly at 2.4 and 4.8 µg/mL concentrations. Pre-treatment with PEFML effectively reduced PQ-induced apoptosis in RAW264.7 macrophages by modulating the Bax/Bcl-2 ratio.

Conclusions: These findings suggest that PEFML exerts a cytoprotective effect against PQ-induced apoptosis in RAW264.7 macrophages, likely through the attenuation of oxidative stress and regulation of apoptotic signaling pathways.

Keywords: apoptosis; *Morus alba* L. leaves; paraquat; polyphenols; RAW264.7 macrophages.

Resumen

Contexto: El paraquat (PQ) induce citotoxicidad principalmente a través de la generación de especies reactivas de oxígeno (ROS) intracelulares, lo que conduce a la apoptosis. Las hojas de *Morus alba* L. son ricas en polifenoles, conocidos por sus potentes propiedades antioxidantes.

Objetivos: Cuantificar el contenido fenólico total, la capacidad antioxidante total y la actividad antioxidante de los extractos de polifenoles de hojas de *Morus alba* L. (PEFML), así como evaluar su efecto protector frente a la citotoxicidad inducida por PQ en macrófagos RAW264.7.

Métodos: Se evaluaron el contenido fenólico total, la capacidad antioxidante total y la actividad antioxidante del PEFML mediante ensayos *in vitro* estándar. Los macrófagos RAW264.7 fueron pretratados con PEFML durante 24 horas y posteriormente expuestos a PQ (0,8 mM) durante otras 24 horas. A continuación, se analizaron la viabilidad celular, la apoptosis, la generación intracelular de ROS y la expresión relativa del mRNA de Bax/Bcl-2.

Resultados: El PEFML demostró una actividad antioxidante significativa, evidenciada por su alto contenido fenólico y elevada capacidad antioxidante. En concentraciones de 1,2 a 9,6 µg/mL, el PEFML no mostró citotoxicidad en las células RAW264.7. En particular, el PEFML suprimió de forma significativa la generación intracelular de ROS, especialmente a concentraciones de 2,4 y 4,8 µg/mL. El pretratamiento con PEFML redujo eficazmente la apoptosis inducida por PQ en macrófagos RAW264.7 mediante la modulación de la relación Bax/Bcl-2.

Conclusiones: Estos resultados sugieren que el PEFML ejerce un efecto citoprotector frente a la apoptosis inducida por PQ en macrófagos RAW264.7, probablemente mediante la atenuación del estrés oxidativo y la regulación de las vías de señalización apoptótica.

Palabras Clave: apoptosis; macrófagos RAW264.7; *Morus alba* L.; paraquat; polifenoles.

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AUTHOR INFO

ORCID:

[0000-0001-6391-4203](https://orcid.org/0000-0001-6391-4203) (PS)

[0000-0003-1317-9436](https://orcid.org/0000-0003-1317-9436) (JW)

[0000-0002-7547-0213](https://orcid.org/0000-0002-7547-0213) (WA)

Abbreviations: ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); Bax: BCL2 associated X; Bcl-2: BCL2 apoptosis regulator; cDNA: complementary deoxyribonucleic acid; DCFDA: 2',7'-dichlorofluorescein diacetate; DNA: deoxyribonucleic acid; FeSO₄: ferrous sulphate; FITC: fluorescein isothiocyanate; FRAP: ferric-reducing antioxidant power; GAE: gallic acid equivalent; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC: N-acetylcysteine; IL: interleukin; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PEFML: polyphenols extracted from *Morus alba* L. leaves; PI: propidium iodide; PQ: paraquat; ROS: reactive oxygen species; RNA: ribonucleic acid; SEM: standard error of the mean; TPTZ: 2,4,6-tripyridyl-s-triazine.

INTRODUCTION

Paraquat (PQ) is a widely used herbicide in agriculture; however, it exhibits high toxicity to both animals and humans. Although the precise molecular mechanism underlying PQ-induced cytotoxicity remains unclear, it is primarily attributed to redox cycling processes that generate excessive intracellular reactive oxygen species (ROS). The accumulation of ROS leads to oxidative stress, mitochondrial dysfunction, and apoptosis (Castello et al., 2007; Chang et al., 2013; Srinontong et al., 2022). Previous studies have demonstrated that PQ increases ROS levels, disrupts mitochondrial membranes, and activates pro-apoptotic signaling pathways (Yang and Tiffany-Castiglioni, 2008; Zhao et al., 2017). Additionally, PQ has been reported to trigger immune responses by inducing cytokine release and inflammation (Liu et al., 2014).

Various antioxidant-based strategies have been proposed to mitigate PQ-induced cytotoxicity. Compounds such as taurine and oyster mushroom extracts have shown hepatoprotective effects against PQ exposure (Danan Jaya et al., 2017), while vitamin E has been found to reduce PQ toxicity in myocardial tissue (Fahim et al., 2013). Moreover, a wide range of natural antioxidants—including naringin, silymarin, edaravone, *Bathysa cuspidata* extracts, α -lipoic acid, pirfenidone, lysine acetylsalicylate, selenium, quercetin, C-phycocyanin, bacosides, and vitamin C—have been investigated for their protective roles against PQ (Blanco-Ayala et al., 2014). For instance, 5-hydroxy-1-methylhydantoin has been shown to alleviate PQ-induced lung damage (Gao et al., 2020a).

Macrophages, which are ubiquitously distributed in tissues, play a crucial role in both innate and adaptive immune responses. They are actively involved in host defense mechanisms and the release of inflammatory mediators such as ROS, nitric oxide (NO), prostaglandins, and pro-inflammatory cytokines, including interleukin (IL)-1 β and IL-6 (Hwang et al., 2019; Wu et al., 2013; Zhang and Wang, 2014). An imbalance in redox homeostasis within these cells can exacerbate oxidative stress and inflammation (Leopold and Loscalzo, 2008; Mills and O'Neill, 2016). Therefore, the discovery of natural antioxidant agents capable of modulating ROS production in macrophages is of significant biomedical interest.

Morus alba L. (white mulberry), a medicinal plant belonging to the *Moraceae* family, has been extensively utilized in traditional medicine across various cultures. Its leaves are rich in bioactive compounds, including alkaloids, flavonoids, stilbenoids, and phenolic acids (Gao et al., 2020b). Numerous pharmacological properties have been attributed to *M. alba* leaves, such as antimicrobial (Cui et al., 2019), anti-obesity (Ann et al., 2015), anti-diabetic (Zhang et al., 2014), hepatoprotective (Liang et al., 2021), and anti-inflammatory activities (Gao et al., 2020b; Lee et al., 2020). Importantly, their antioxidant potential has been demonstrated in several studies, suggesting their efficacy in protecting cells from oxidative damage (Doi et al., 2000; Liang et al., 2021). Given that PQ exerts its toxicity through excessive ROS generation, it is pertinent to explore the protective effects of *M. alba* leaves against PQ-induced cytotoxicity.

To date, limited research has explored the use of polyphenolic compounds—natural antioxidants derived from *M. alba* leaves—in mitigating PQ toxicity. The current study hypothesized that polyphenol-enriched fractions from *M. alba* leaves (PEFML) could attenuate PQ-induced cytotoxicity and improve cell viability in RAW264.7 macrophages. Furthermore, this study aimed to elucidate the protective mechanism of PEFML by investigating its effect on intracellular ROS levels and the Bax/Bcl-2 apoptotic ratio.

This work represents the first investigation of the cytoprotective effects of PEFML against PQ-induced apoptosis in RAW264.7 macrophages. Specifically, the objectives were to evaluate the total polyphenol content, antioxidant capacity, and biological activity of PEFML, as well as to assess its ability to counteract PQ-induced oxidative stress and apoptotic cell death. The findings are expected to contribute to the validation of traditional medicinal uses of *M. alba* and highlight its potential as a natural therapeutic agent for the management of ROS-mediated cytotoxicity.

MATERIAL AND METHODS

Experimental design

The study was conducted in two main phases. The first phase involved the characterization of *M. alba* leaf extracts through the determination of total polyphenol content, ferric-reducing antioxidant power (FRAP), and free radical scavenging activity using the

ABTS assay. These analyses aimed to evaluate the extract's suitability for further experimentation. The second phase consisted of three sub-studies: (1) evaluation of the cytoprotective efficacy of polyphenol extracts from *M. alba* leaves (PEFML) against paraquat (PQ)-induced toxicity in RAW264.7 macrophages; (2) investigation of the effect of PEFML on PQ-induced reactive oxygen species (ROS) production; and (3) examination of the anti-apoptotic effects of PEFML through modulation of mitochondrial apoptotic signaling.

Chemicals

Gallic acid was purchased from Fluka (Steinheim, Germany), acetate buffer (pH 3.6), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ from Ajax Finechem Pty. Ltd. (Taren Point, Australia). Paraquat (PQ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), fetal bovine serum, and N-acetylcysteine (NAC) (Sigma-Aldrich, St. Louis, MO, USA). HCl (QR $\text{\textcircled{R}}$ C TM , Auckland, New Zealand). Dimethylsulphoxide (DMSO) and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (PanReac AppliChem, Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Cytiva, USA). Penicillin-streptomycin from Gibco BRL (Life Technologies, USA). DCFDA/H $_2$ DCFDA ROS assay kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Abcam, USA). FITC Annexin V Apoptosis Detection Kit with PI (BioLengend, San Diego, CA). Nucleospin RNA kit (Macherey-Nagel, Germany). ReverTra Ace qPCR RT Kit (Toyobo, Japan).

Plant material

Fresh *M. alba* leaves were collected in July 2020 from the botanical garden of the Faculty of Veterinary Sciences, Mahasarakham University, Thailand (GPS coordinates: 16.2459° N, 103.2502° E). Botanical identification was verified by the Center of Excellence in Silk Innovation, Mahasarakham University. The leaves were dried at 60 °C for 6 h and ground into a fine powder. Extraction was performed using 95% ethanol. The extract was filtered, concentrated by rotary evaporation, and subsequently freeze-dried for use in downstream analyses.

Total polyphenol content

Total phenolic content was quantified using the Folin-Ciocalteu colorimetric method as described by Um et al. (2017). Briefly, the extract was prepared and mixed with 10% Folin-Ciocalteu reagent and 7% sodium carbonate solution at a ratio of 2:10:8 and left in the dark for 30 min. Absorbance was measured at 700 nm using a microplate reader (TECAN, Tecan Trading AG, Switzerland). Gallic acid was used as the calibration standard, and results were expressed in

milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

Ferric reducing antioxidant power (FRAP) assay

Antioxidant capacity was assessed via the FRAP method, following the modified protocol of Benzie and Strain (1996). The FRAP working reagent consisted of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a 10:1:1 ratio. A volume of 500 μL of PEFML was incubated with 7.5 mL of FRAP reagent at 37 °C for 6 min, and absorbance was measured at 593 nm. FRAP values were expressed as mM Fe^{2+} equivalents per gram of sample.

ABTS radical scavenging capacity

The ABTS assay was performed following a modified method by Bécquer-Viart et al. (2018). The $\text{ABTS}^+ \cdot$ radical solution was generated by incubating a 1:1 mixture of 7 mM ABTS and 2.45 mM potassium persulfate in the dark for 16 h. The solution was diluted with distilled water to an absorbance of 0.700 ± 0.02 at 734 nm. PEFML (20 μL) was added to 200 μL of the ABTS working solution and incubated for 6 min at room temperature in the dark. Absorbance was measured at 734 nm, and antioxidant activity was expressed as $\mu\text{g}/\text{mL}$ using an ascorbic acid standard curve.

In vitro studies

Cell culture

RAW264.7 mouse macrophages were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

Cell viability assay

Cell viability was assessed using the MTT assay. RAW264.7 cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well and incubated for 24 h. Cells were then treated with various concentrations of PEFML, PQ, or PEFML with PQ for the indicated period. NAC (0.5 mM) was used as a positive control for antioxidant protection. After treatment, 20 μL of MTT solution (5 mg/mL) was added and incubated for 4 h. The medium was discarded, and 150 μL of DMSO was added to solubilize formazan crystals. Absorbance was read at 570 nm.

Apoptosis assay

Apoptosis was evaluated using the FITC Annexin V/PI apoptosis detection kit. Cells were pretreated

with PEFML for 24 h and subsequently exposed to 0.8 mM PQ for 24 h. After treatment, cells were stained with Annexin V and PI for 15 min at room temperature in the dark and analyzed via flow cytometry (FACSCelesta, BD Biosciences, NJ, USA) using FlowJo software.

Measurement of intracellular ROS

Intracellular ROS levels were determined using the DCFDA/H₂DCFDA ROS assay kit. Cells were treated with PEFML (0.6–4.8 µg/mL) for 24 h and then exposed to 0.8 mM PQ 24 h. Following incubation with 20 µM DCFH-DA at 37°C for 30 min, cells were washed and resuspended in PBS. Fluorescence intensity was measured by flow cytometry.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the Nucleospin RNA kit. RNA concentration and purity were assessed with a Nanodrop 2000 spectrophotometer. cDNA synthesis was performed with ReverTra Ace qPCR RT kit. qRT-PCR was conducted using Thunderbird SYBR qPCR Master Mix on a QuantStudio™ 3 Real-Time PCR System. GAPDH was used as a housekeeping gene. Primer sequences are listed in Table 1. Relative expression levels were normalized to GAPDH. The thermal cycling conditions were run as follows: pre-denaturation at 95°C for 5 min, 1 cycle, followed by 40 cycles at 95°C for 15 s, annealing temperature at 60°C for 15 s, and extension at 72°C for 20 s. The fold-change in gene expression was determined using the 2^{−ΔΔCT} method. Then, the Bax/Bcl-2 ratio was calculated.

Statistical analysis

For all experiments, data were analyzed using Excel and expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was applied, followed by Duncan's multiple range test for post hoc comparisons. Statistical significance was defined as $p < 0.05$.

RESULTS

Total polyphenol content, ferric-reducing antioxidant power (FRAP), and ABTS radical scavenging capacity of *M. alba* leaf extracts (PEFML)

The total polyphenol content, FRAP value, and ABTS radical scavenging activity of PEFML were found to be 11.56 ± 2.04 mg GAE/g extract, 14.93 ± 1.16 mM Fe²⁺/g extract, and 59.72 ± 18.69 µg/mL ascorbic acid equivalents, respectively (Table 2).

Cytotoxicity of PEFML in RAW264.7 macrophages

The cytotoxicity of PEFML at concentrations of 0.6, 1.2, 2.4, 4.8, and 9.6 µg/mL was evaluated after 24 and 48 hours of exposure. At 24 hours, cell viability was significantly increased at concentrations of 1.2–9.6 µg/mL compared to the control group ($p < 0.05$). At 48 hours, significant increases in viability were observed at 1.2–4.8 µg/mL ($p < 0.05$). Furthermore, PEFML exhibited a proliferative effect on RAW264.7 cells at concentrations of 1.2–4.8 µg/mL across both time points (Fig. 1A-B).

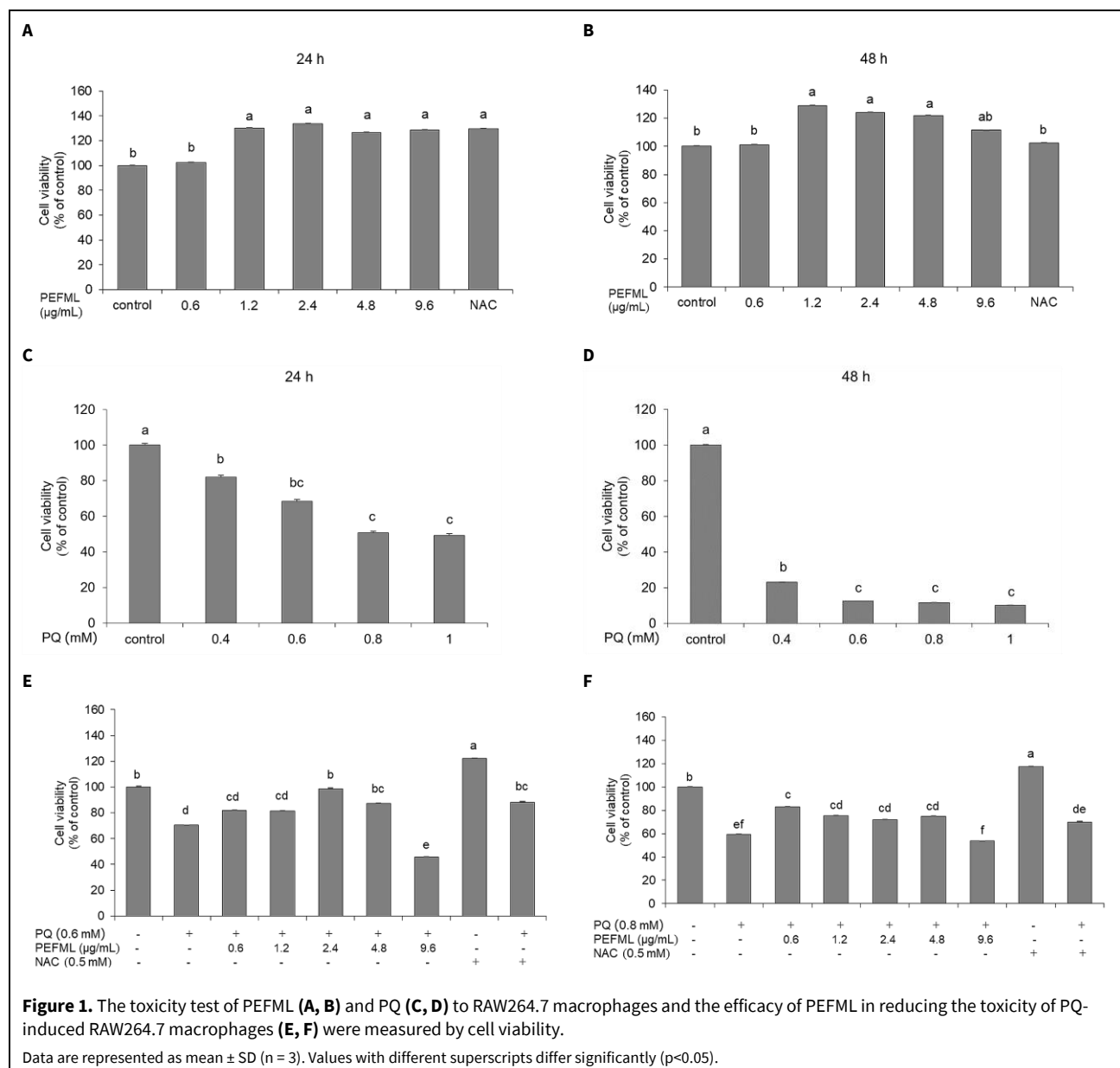
Table 1. Primer sequences used for the real-time quantitative PCR.

Gene	Primer sequences (5' to 3')	Reference
<i>Bcl-2</i>	F: CCGGGAGAACAGGTATGATAA R: CCCACTCGTAGCCCTCTG	Hu et al., 2013
<i>Bax</i>	F: ATGCGTCCACCAAGAAGCTGA R: AGCAATCATCCTCTGCAGCTCC	Hu et al., 2013
GAPDH	F: GGCATTGTGGAAGGGCTCAT R: GACACATTGGGGGTAGGAACAC	

Table 2. Total polyphenols, FRAP, and ABTS scavenging activity of *Morus alba* L. leaf extracts.

Parameters	Mean ± SEM
Total polyphenols (mg GAE/g extract)	11.56 ± 2.04
FRAP (mM Fe ²⁺ /g extract)	14.93 ± 1.16
ABTS (µg/mL ascorbic acid)	59.72 ± 18.69

GAE: gallic acid equivalent; FRAP: ferric-reducing antioxidant power; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); SEM: standard error of the mean



Cytotoxicity of paraquat in RAW264.7 macrophages

Exposure of RAW264.7 macrophages to PQ at concentrations of 0.4–1.0 mM for 24 and 48 hours significantly reduced cell viability compared to untreated controls ($p < 0.05$). Notably, PQ at 0.6 and 0.8 mM reduced cell viability by approximately 68% and 50%, respectively, and these concentrations were subsequently selected for further experimentation (Fig. 1C–D).

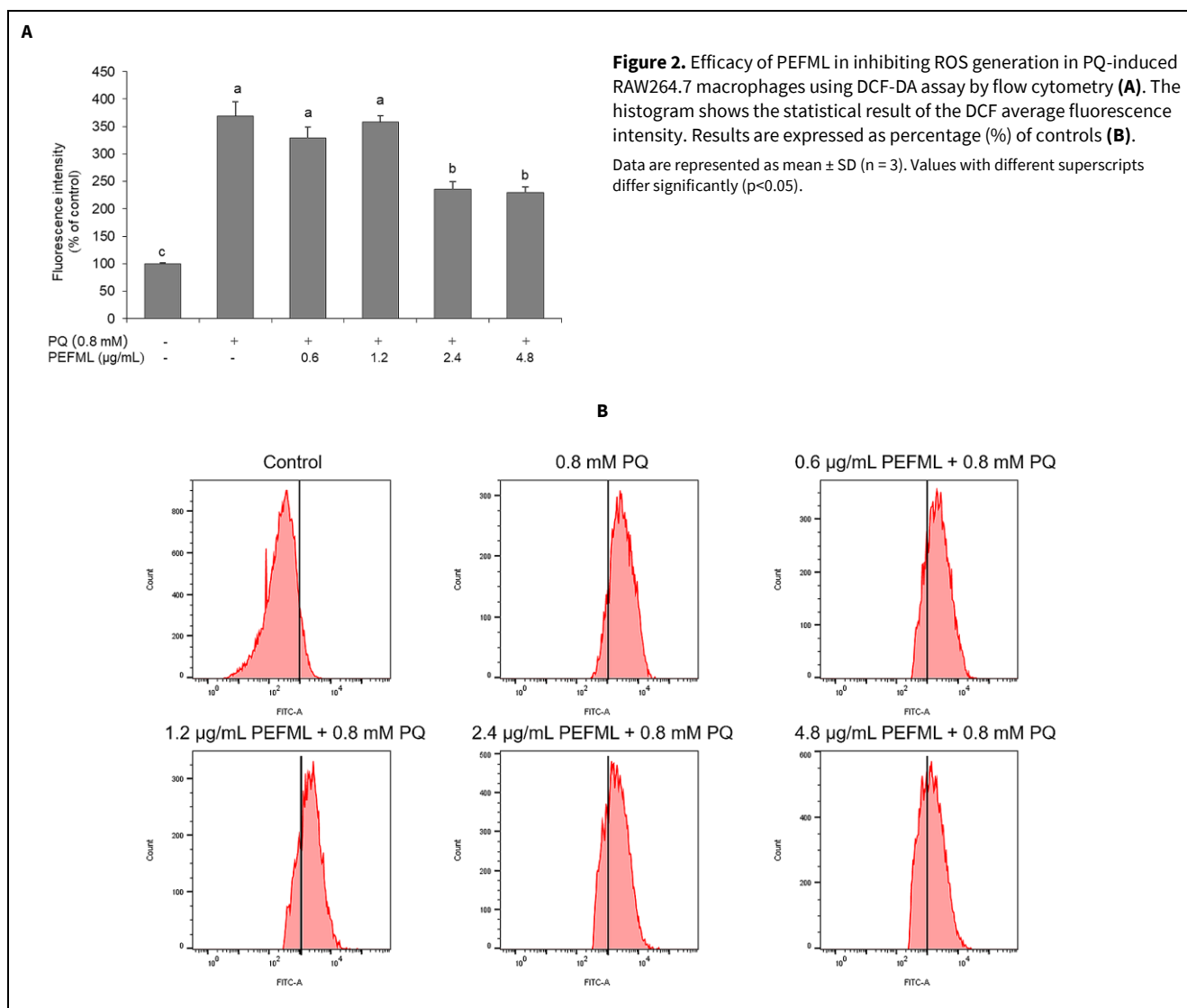
Protective effect of PEFML against PQ-induced cytotoxicity

Pre-treatment of RAW264.7 macrophages with PEFML (0.6–9.6 μg/mL) for 24 hours, followed by exposure to 0.6 mM PQ for an additional 24 hours, resulted in significantly higher cell viability at 2.4 and

4.8 μg/mL PEFML compared to the PQ-only group ($p < 0.05$). When cells were exposed to 0.8 mM PQ, pre-treatment with PEFML at concentrations of 0.6–4.8 μg/mL also significantly improved viability ($p < 0.05$), comparable to the effect observed with NAC treatment. However, PEFML at 9.6 μg/mL led to a reduction in cell viability under both PQ exposure levels (Fig. 1E–F).

Effect of PEFML on PQ-induced intracellular ROS generation

ROS production was significantly elevated in RAW264.7 macrophages following treatment with 0.8 mM PQ compared to controls ($p < 0.05$). Pre-treatment with PEFML at 2.4 and 4.8 μg/mL significantly attenuated PQ-induced ROS generation ($p < 0.05$) (Fig. 2).



Anti-apoptotic effect of PEFML in PQ-treated RAW264.7 macrophages

Flow cytometry analysis using Annexin V-FITC/PI staining revealed that PQ significantly increased the percentage of apoptotic cells compared to untreated controls ($p < 0.05$). Pre-treatment with PEFML at 1.2 and 4.8 $\mu\text{g/mL}$ significantly reduced apoptosis levels ($p < 0.05$) (Fig. 3A-B).

Quantitative RT-PCR analysis indicated that the Bax/Bcl-2 ratio was significantly elevated in PQ-treated cells compared to the control group ($p < 0.05$). Pre-treatment with PEFML at 2.4 and 4.8 $\mu\text{g/mL}$ significantly reduced the Bax/Bcl-2 ratio ($p < 0.05$), restoring levels comparable to the control group ($p > 0.05$) (Fig. 3C).

DISCUSSION

Natural products are increasingly being investigated for their potential to mitigate the toxic effects of harmful substances, such as PQ, a widely used but

highly toxic herbicide. The primary mechanism of PQ-induced toxicity involves the generation of ROS, leading to oxidative stress and subsequent cellular damage (Chang et al., 2013; Wu et al., 2018). In the present study, PEFML, known for its antioxidant potential (Chanhan et al., 2019; Yu et al., 2018), was evaluated for its protective effects against PQ-induced cytotoxicity in RAW264.7 macrophages.

The PEFML total polyphenol content was found to be 11.56 ± 2.04 mg GAE/g extract, consistent with previous reports that have described values ranging from 10 to 20 mg/g (Chanhan et al., 2019; Iqbal et al., 2012; Sánchez-Salcedo et al., 2015). The ferric-reducing antioxidant power (FRAP) was 14.93 ± 1.16 mM Fe^{2+} /g extract, and the ABTS radical scavenging activity was 59.72 ± 18.69 $\mu\text{g/mL}$ ascorbic acid equivalents—both values aligning with earlier findings (Chanhan et al., 2019; Cui et al., 2019; Peanparkdee et al., 2016).

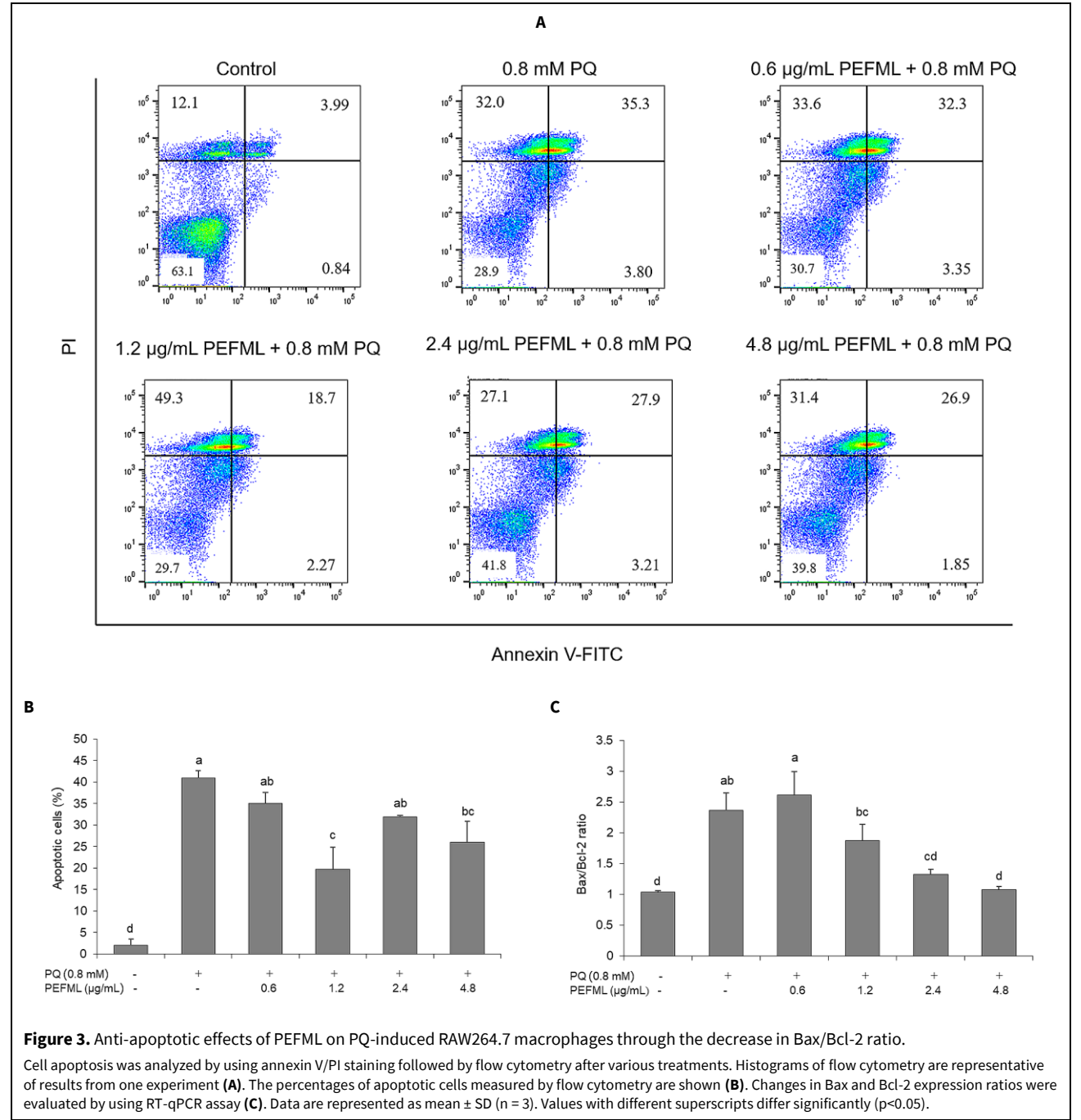
The cytotoxicity of PEFML on RAW264.7 macrophages was subsequently assessed. PEFML at concen-

trations of 1.2–9.6 µg/mL (24 h) and 1.2–4.8 µg/mL (48 h) significantly increased cell viability, indicating non-toxicity and a potential proliferative effect. These results are consistent with those of Soonthornsit et al. (2017), Tseng et al. (2018), and Yu et al. (2021), who reported that polyphenols derived from *M. alba* roots and fruits were not cytotoxic to RAW264.7 macrophages. This study is the first to report that leaf-derived polyphenols from *M. alba* similarly do not compromise macrophage viability.

To establish a toxic baseline, RAW264.7 macrophages were exposed to PQ at various concentrations. PQ significantly reduced cell viability at 0.4–1.0 mM

after both 24 and 48 h of treatment, consistent with previous studies describing its pro-apoptotic and cytotoxic effects via ROS overproduction (Castello et al., 2007; Chang et al., 2013; Zhao et al., 2017).

Pre-treatment with PEFML significantly increased cell viability in PQ-exposed RAW264.7 macrophages. The extent of protection depended on the concentration of both PEFML and PQ. These findings align with Liang et al. (2021), who demonstrated that *M. alba* extracts protected hepatocytes against acetaldehyde-induced cytotoxicity. This is the first study to report the protective effects of PEFML against PQ-induced cytotoxicity in RAW264.7 macrophages.



The generation of intracellular ROS is a critical mediator of PQ-induced cytotoxicity (Orrenius et al., 2007; Srinontong et al., 2022). In this study, a significant increase in intracellular ROS levels was observed following PQ exposure. Pre-treatment with PEFML at concentrations of 2.4 and 4.8 µg/mL effectively reduced ROS generation. This effect corroborates findings by Carvalho and Cerdeira (2016), who reported that *M. alba* extracts decreased ROS in LPS-induced RAW264.7 cells and activated neutrophils.

Given that mitochondria are central to both ROS generation and apoptosis signaling (Orrenius et al., 2007), their role in PQ-induced cytotoxicity is crucial. Mitochondrial dysfunction caused by excessive ROS triggers apoptosis through the intrinsic pathway, primarily regulated by pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins (Fuchs and Steller, 2011). PQ has been shown to increase Bax expression, reduce Bcl-2, and activate caspase-dependent apoptosis (Liu et al., 2018).

In the present study, PQ exposure significantly increased the Bax/Bcl-2 ratio and apoptosis rate in RAW264.7 macrophages. Pre-treatment with PEFML resulted in a marked reduction in both apoptosis and the Bax/Bcl-2 ratio, suggesting that PEFML confers cytoprotection via modulation of mitochondrial apoptotic signaling. These findings are consistent with previous evidence indicating that *M. alba* extracts can attenuate oxidative stress-induced apoptosis in hepatocytes (Liang et al., 2021).

CONCLUSION

The findings of this study demonstrate that *Morus alba* L. leaves are a rich source of polyphenols with potent antioxidant activity. PQ, a known pro-oxidant agent, was used to induce cytotoxicity in RAW264.7 macrophages, resulting in elevated intracellular ROS levels and oxidative stress. Treatment with polyphenol-enriched extracts from *M. alba* leaves (PEFML) significantly mitigated PQ-induced cytotoxicity in macrophages.

The protective mechanism of PEFML was associated with the attenuation of intracellular ROS production and downregulation of the Bax/Bcl-2 ratio, both of which are key regulators of the mitochondrial apoptotic pathway. Furthermore, optimal concentrations of PEFML not only exhibited no cytotoxicity but also promoted macrophage viability, indicating potential immunomodulatory benefits.

These results suggest that polyphenols derived from *M. alba* leaves may serve as promising natural agents for mitigating PQ-induced oxidative damage. However, as *M. alba* leaf extracts contain a variety of polyphenolic compounds, further investigations are

warranted to isolate and characterize individual active constituents. Such studies would contribute to elucidating the specific mechanisms of action and support the development of targeted applications for *M. alba* as a therapeutic or preventive agent against oxidative stress-related cytotoxicity.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

- Ann JY, Eo H, Lim Y (2015) Mulberry leaves (*Morus alba* L.) ameliorate obesity-induced hepatic lipogenesis, fibrosis, and oxidative stress in high-fat diet-fed mice. *Genes Nutr* 10: 46. <https://doi.org/10.1007/s12263-015-0495-x>
- Bécquer-Viart MA, González-Yaque J, Fonseca-Fonseca LA, Núñez-Figueroa Y, Pardo Andreu GL (2018) Antioxidant and neuroprotective effects of gossypitrin, a flavonoid from *Talipariti elatum*, against chemical hypoxia-induced PC12 cell death. *J Pharm Pharmacogn Res* 6(2): 72–80. https://doi.org/10.56499/jppres17.314_6.2.72
- Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal Biochem* 239(1): 70–76. <https://doi.org/10.1006/abio.1996.0292>
- Blanco-Ayala T, Andérica-Romero A, Pedraza-Chaverri J (2014) New insights into antioxidant strategies against paraquat toxicity. *Free Radic Res* 48(6): 623–640. <https://doi.org/10.3109/10715762.2014.899694>
- Carvalho L, Cerdeira C (2016) Modulatory effects of the mulberry leaf extract on reactive oxygen species generation during the neutrophil respiratory burst. *J Sci* 6(9): 435–441.
- Castello PR, Drechsel DA, Patel M (2007) Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem* 282(19): 14186–14193. <https://doi.org/10.1074/jbc.M700827200>
- Chang X, Lu W, Dou T, Wang X, Lou D, Sun X, Zhou Z (2013) Paraquat inhibits cell viability via enhanced oxidative stress and apoptosis in human neural progenitor cells. *Chem Biol Interact* 206(2): 248–255. <https://doi.org/10.1016/j.cbi.2013.09.010>
- Chanhan P, Konsue A, Nammatra R (2019) Effect of agricultural model of using fertilizer, harvesting time and extraction method on phytochemical contents and antioxidant activities from mulberry leaves grown in Maha Sarakham Province, Thailand. *Pharmacogn J* 11(3): 531–535. <https://doi.org/10.5530/pj.2019.11.85>
- Cui H, Lu T, Wang M, Zou X, Zhang Y, Yang X, Dong Y, Zhou H (2019) Flavonoids from *Morus alba* L. leaves: Optimization of extraction by response surface methodology and comprehensive evaluation of their antioxidant, antimicrobial, and inhibition of α-amylase activities through analytical hierarchy process. *Molecules* 24(13): 2398. <https://doi.org/10.3390/molecules24132398>

- Danan Jaya BP, Widiastuti EL, Nurcahyani E, Sutyarso S (2017) Taurine and oyster mushroom (*Pleurotus ostreatus*) prevents oxidative damage in liver of mice induced by paraquat. *Biomed Pharmacol J* 10(4): 1993–2000. <https://dx.doi.org/10.13005/bpj/1320>
- Doi K, Kojima T, Fujimoto Y (2000) Mulberry leaf extract inhibits the oxidative modification of rabbit and human low density lipoprotein. *Biol Pharm Bull* 23(9): 1066–1071. <https://doi.org/10.1248/bpb.23.1066>
- Fahim MA, Howarth FC, Nemmar A, Qureshi MA, Shafiullah M, Jayaprakash P, Hasan MY (2013) Vitamin E ameliorates the decremental effect of paraquat on cardiomyocyte contractility in rats. *PLoS One* 8(3): e57651. <https://doi.org/10.1371/journal.pone.0057651>
- Fuchs Y, Steller H (2011) Programmed cell death in animal development and disease. *Cell* 147(4): 742–758. <https://doi.org/10.1016/j.cell.2011.10.033>
- Gao L, Yuan H, Xu E, Liu J (2020a) Toxicology of paraquat and pharmacology of the protective effect of 5-hydroxy-1-methylhydantoin on lung injury caused by paraquat based on metabolomics. *Sci Rep* 10: 1790. <https://doi.org/10.1038/s41598-020-58599-y>
- Gao XH, Zhang SD, Wang LT, Yu L, Zhao XL, Ni HY, Wang YQ, Wang JD, Shan CH, Fu YJ (2020b) Anti-inflammatory effects of neochlorogenic acid extract from mulberry leaf (*Morus alba* L.) against LPS-stimulated inflammatory response through mediating the AMPK/Nrf2 signaling pathway in A549 cells. *Molecules* 25(6): 1385. <https://doi.org/10.3390/molecules25061385>
- Hu W, Ge Y, Ojcius DM, Sun D, Dong H, Yang XF, Yan J (2013) p53 signalling controls cell cycle arrest and caspase-independent apoptosis in macrophages infected with pathogenic *Leptospira* species. *Cell Microbiol* 15(10): 1624–1659. <https://doi.org/10.1111/cmi.12141>
- Hwang JH, Ma JN, Park JH, Jung HW, Park YK (2019) Anti-inflammatory and antioxidant effects of MOK, a polyherbal extract, on lipopolysaccharide-stimulated RAW264.7 macrophages. *Int J Mol Med* 43(1): 26–36. <https://doi.org/10.3892/ijmm.2018.3937>
- Iqbal S, Younas U, Chan KW, Sarfraz RA, Uddin M (2012) Proximate composition and antioxidant potential of leaves from three varieties of mulberry (*Morus* sp.): A comparative study. *Int J Mol Sci* 13(6): 6651–6664. <https://doi.org/10.3390/ijms13066651>
- Lee MR, Kim JE, Park JJ, Choi JY, Song BR, Choi YW, Kim DS, Kim KM, Song HK, Hwang DY (2020) Protective role of fermented mulberry leave extract in LPS-induced inflammation and autophagy of RAW264. 7 macrophage cells. *Mol Med Rep* 22(6): 4685–4695. <https://doi.org/10.3892/mmr.2020.11563>
- Leopold JA, Loscalzo J (2008) Oxidative mechanisms and atherothrombotic cardiovascular disease. *Drug Discov Today Ther Strateg* 5(1): 5–13. <https://doi.org/10.1016/j.ddstr.2008.02.001>
- Liang HW, Yang TY, Teng CS, Lee YJ, Yu MH, Lee HJ, Hsu LS, Wang CJ (2021) Mulberry leaves extract ameliorates alcohol-induced liver damages through reduction of acetaldehyde toxicity and inhibition of apoptosis caused by oxidative stress signals. *Int J Med Sci* 18(1): 53–64. <https://doi.org/10.7150/ijms.50174>
- Liu Z, Sun M, Wang Y, Zhang L, Zhao H, Zhao M (2018) Silymarin attenuated paraquat-induced cytotoxicity in macrophage by regulating Trx/TXNIP complex, inhibiting NLRP3 inflammasome activation and apoptosis. *Toxicol In Vitro* 46: 265–272. <https://doi.org/10.1016/j.tiv.2017.10.017>
- Liu Z, Wang Y, Zhao H, Zheng Q, Xiao L, Zhao M (2014) CB2 receptor activation ameliorates the proinflammatory activity in acute lung injury induced by paraquat. *Biomed Res Int* 2014: 971750. <https://doi.org/10.1155/2014/971750>
- Mills EL, O'Neill LA (2016) Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *Eur J Immunol* 46(1): 13–21. <https://doi.org/10.1002/eji.201445427>
- Orrenius S, Gogvadze V, Zhivotovsky B (2007) Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol* 47: 143–183. <https://doi.org/10.1146/annurev.pharmtox.47.120505.105122>
- Peanparkdee M, Iwamoto S, Borompichaichartkul C, Duangmal K, Yamauchi R (2016) Microencapsulation of bioactive compounds from mulberry (*Morus alba* L.) leaf extracts by protein-polysaccharide interactions. *Int J Food Sci Technol* 51(3): 649–655. <https://doi.org/10.1111/ijfs.13032>
- Sánchez-Salcedo EM, Mena P, García-Viguera C, Hernández F, Martínez JJ (2015) (Poly) phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals. *J Funct Foods* 18(Part B): 1039–1046. <https://doi.org/10.1016/j.jff.2015.03.053>
- Soonthornsit N, Pitaksutheepong C, Hemstapat W, Utasincharnoen P, Pitaksutheepong T (2017) *In vitro* anti-inflammatory activity of *Morus alba* L. stem extract in LPS-stimulated RAW264.7 cells. *Evid Based Complement Alternat Med* 2017: 3928956. <https://doi.org/10.1155/2017/3928956>
- Srinontong P, Wandee J, Angwanich W (2022) Paraquat modulates immunological function in bone marrow-derived macrophages. *Acta Vet Hung* 70(1): 64–72. <https://doi.org/10.1556/004.2022.00003>
- Tseng TH, Lin WL, Chang CK, Lee KC, Tung SY, Kuo HC (2018) Protective effects of morus root extract (MRE) against lipopolysaccharide-activated RAW264. 7 cells and CCl4-induced mouse hepatic damage. *Cell Physiol Biochem* 51(3): 1376–1388. <https://doi.org/10.1159/000495555>
- Um M, Shin GJ, Lee JW (2017) Extraction of total phenolic compounds from yellow poplar hydrolysate and evaluation of their antioxidant activities. *Ind Crops Prod* 97: 574–581. <https://doi.org/10.1016/j.indcrop.2016.12.062>
- Wu J, Yan Z, Schwartz DE, Yu J, Malik AB, Hu G (2013) Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury. *J Immunol* 190(7): 3590–3599. <https://doi.org/10.4049/jimmunol.1200860>
- Wu Q, Xu Q, Jian X, Wang H, He X, Gao B, Wang K, Kan B (2018) A new sight for paraquat poisoning from immunology. *Immunopharmacol Immunotoxicol* 40(4): 269–272. <https://doi.org/10.1080/08923973.2018.1490319>
- Yang W, Tiffany-Castiglioni E (2008) Paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells: involvement of p53 and mitochondria. *J Toxicol Environ Health A* 71(4): 289–299. <https://doi.org/10.1080/15287390701738467>
- Yu JS, Lim SH, Lee SR, Choi C-I, Kim KH (2021) Antioxidant and anti-inflammatory effects of white mulberry (*Morus alba* L.) fruits on lipopolysaccharide-stimulated RAW264.7 macrophages. *Molecules* 26(4): 920. <https://doi.org/10.3390/molecules26040920>
- Yu Y, Li H, Zhang B, Wang J, Shi X, Huang J, Yang J, Zhang Y, Deng Z (2018) Nutritional and functional components of mulberry leaves from different varieties: Evaluation of their potential as food materials. *Int J Food Prop* 21(1): 1495–1507. <https://doi.org/10.1080/10942912.2018.1489833>
- Zhang L, Wang CC (2014) Inflammatory response of macrophages in infection. *Hepatobiliary Pancreat Dis Int* 13(2): 138–152. [https://doi.org/10.1016/s1499-3872\(14\)60024-2](https://doi.org/10.1016/s1499-3872(14)60024-2)

Zhang Y, Ren C, Lu G, Mu Z, Cui W, Gao H, Wang Y (2014) Anti-diabetic effect of mulberry leaf polysaccharide by inhibiting pancreatic islet cell apoptosis and ameliorating insulin secretory capacity in diabetic rats. *Int Immunopharmacol* 22(1): 248–257. <https://doi.org/10.1016/j.intimp.2014.06.039>

Zhao G, Cao K, Xu C, Sun A, Lu W, Zheng Y, Li H, Hong G, Wu B, Qiu Q, Lu Z (2017) Crosstalk between mitochondrial fission and oxidative stress in paraquat-induced apoptosis in mouse alveolar type II cells. *Int J Biol Sci* 13(7): 888–900. <https://doi.org/10.7150/ijbs.18468>

AUTHOR CONTRIBUTION:

Contribution	Wandee J	Srinontong P	Aengwanich W	Srisanyong W
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content		x		
Literature search		x		
Experimental studies	x	x	x	
Data acquisition	x	x	x	
Data analysis	x	x		
Statistical analysis		x		
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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