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Effects of *Pogonatherum paniceum* (Lamk) Hack extract on anti-mitochondrial DNA mediated inflammation by attenuating *Tlr9* expression in LPS-induced macrophages

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ABSTRACT

BACKGROUND/OBJECTIVES: Mitochondrial DNA leakage leads to inflammatory responses via endosome activation. This study aims to evaluate whether the perennial grass water extract (*Pogonatherum panicum*) ameliorate mitochondrial DNA (mtDNA) leakage.
MATERIALS/METHODS: The major bioactive constituents of *P. paniceum* (PPW) were investigated by high-performance liquid chromatography, after which their antioxidant activities were assessed. In addition, RAW 264.7 macrophages were stimulated with lipopolysaccharide, resulting in mitochondrial damage. Quantitative polymerase chain reaction and enzyme-linked immunosorbent assay were used to examine the gene expression and cytokines.
RESULTS: Our results showed that PPW extract-treated activated cells significantly decrease reactive oxygen species and nitric oxide levels by reducing the $p22^{phox}$ and *iNOS* expression and lowering cytokine-encoding genes, including *IL-6*, *TNF-α*, *IL-1β*, *PG-E2* and *IFN-γ* relative to the lipopolysaccharide (LPS)-activated macrophages. Furthermore, we observed that LPS enhanced the mtDNA leaked into the cytoplasm, increasing the transcription of *Tlr9* and signaling both *MyD88*/*Irf7*-dependent interferon and MyD88/NF-κb p65-dependent inflammatory cytokine mRNA expression but which was alleviated in the presence of PPW extract.
CONCLUSIONS: Our data show that PPW extract has antioxidant and anti-inflammatory activities by facilitating mtDNA leakage and lowering the *Tlr9* expression and signaling activation.

Keywords: Medicinal plants; antioxidants; inflammation; mitochondrial DNA; endosome

INTRODUCTION

Oxidative stress is generated by the elevated exhibition of reactive oxygen species (ROS), including hydrogen peroxide, superoxide radicals, peroxynitrite and hydroxyl radicals [1]. Furthermore, a decrease in the antioxidant system leads to increased cellular and molecular

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Conflit of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Thim-uam A, Thongboontho R; Formal analysis: Thim-uam A, Thongboontho R, Petcharat K; Funding acquisition: Thim-uam A, Thongboontho R, Phromnoi K; Investigation: Thim-uam A, Thongboontho R; Methodology: Thim-uam A, Thongboontho R, Petcharat K, Munkong N, Khonthun C, Boondech A, Phromnoi K; Supervision: Thim-uam A; Writing – original draft: Thim-uam A; Writing – review & editing: Thim-uam A, Thongboontho R.

damage, resulting in tissue injury [2,3]. It is well-established that ROS are produced by various cellular compartments, including mitochondria, the cytosol, the cell membrane, the endoplasmic reticulum and peroxisomes [4]. However, mitochondrial oxidative metabolism is a major source of ROS production [5,6]. Moreover, ROS-induced mitochondrial damage promotes the release of double stranded double stranded DNA (dsDNA) to the cytosol [6] and becomes a crucial driver of inflammation [7,8] via DNA sensor pathways, leading to the inflammatory response [9]. Previous evidence established that the mitochondrial DNA (mtDNA) leakage could activate Toll-like receptor 9 (TLR9) in the endosome [10], leading to an inflammatory response via the initiation of myeloid differentiation primary response 88 (MYD88) adaptor protein [11].

Disruption of the antioxidant system can promote the development of chronic inflammation. Cellular antioxidant enzymes such as catalase and superoxide dismutase are naturally occurring mechanisms to regulate ROS levels [12,13]. While several synthetic drugs have been used against oxidative stress and inhibit the inflammatory process, many studies have found them to have adverse toxic effects [14]. Therefore, the antioxidant and anti-inflammatory properties of bioactive compounds in medicinal plants and natural extracts have been studied extensively for the prevention and treatment of various inflammatory diseases.

Polyphenols and flavonoids are major natural compounds present in many plants. Several studies have found such compounds extracted from herbal plants and functional foods to inhibit the excessive ROS production by donating an electron or hydrogen atom, suppressing ROS formation [15,16]. Phenolic compounds are known to reduce oxidative stress and the inflammatory process by suppressing several transcription factors proteins that induce the production of pro-inflammatory cytokines and chemokines such as nuclear factor kappa B (NF-κB) [17,18], mitogen-activated protein kinase (MAPK) [19,20] and activator protein 1 (AP-1) [21]. Interestingly, polyphenols can inhibit type I interferon production in activated macrophages, which plays a crucial role in chronic inflammation by activating the DNA sensing pathway [22].

Among many medicinal plants, *Pogonatherum paniceum* (Lamk) Hack is a perennial grass that has been used in traditional medicine in Southern China, India, and Southeast Asia [23,24]. However, no studies have explored its bioactive compounds and their biological effects, and its underlying mechanism of action remains poorly understood. Therefore, this study explores the major polyphenolic components and the antioxidant and anti-inflammatory properties through TLR9 endosome activation of *P. paniceum* water extract for potential health benefits.

MATERIALS AND METHODS

Preparation of *P. paniceum* extracts (PP)

PP plants were collected in Wang Yang district, Khlongkhlung, Kamphaeng Phet, Thailand. A voucher specimen code No. 0005466 has been deposited at the Department of Thai Traditional and Alternative Medicine, Ministry of Public Health, Thailand. PP water (PPW) extract was prepared as follows. First, PP was dried at 60°C for 12 h in an incubator. Next, 125 g of dried PP was mixed in 3.2 L deionized water (diH_2O) at 70–80°C for 60 min. Then, the mixtures were filtered three times using vacuum filtering apparatus through Whatman filter paper (GE Healthcare, Amersham, UK). Next, PPW extracts were concentrated and lyophilized using a freeze-dryer (Beta 1-8 LSCbasic; Martin Christ, Osterode am Harz,

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Germany). The extraction yield of PPW was 12.03%. Finally, PPW extracts were stored at -80°C prior until required.

Total flavonoids determination

The total flavonoids of PPW were analyzed using the colorimetric assay. Briefly, 250 µL extract was incubated with 75 µL of 5% sodium nitrite ($NaNO_2$) and 1.25 mL distilled water (dH_2O) in the dark for 6 minutes at room temperature (RT). Next, 150 µL of 10% aluminum chloride ($AlCl_3$) was added and incubated at RT for 5 min in the dark. A 500 µL of 1 M sodium hydroxide (NaOH) and 275 µL of dH_2O were added to the mixtures. Finally, the absorbance of reaction mixtures at 510 nm was measured with a UV-visible spectrophotometer (Mecasys Optizen POP; Metertech, Taipei, Taiwan). The total flavonoid was calculated and presented as mg catechin/g extract.

Total phenolic content determination

The total phenolic contents of PPW extracts were analyzed by the Folin-Ciocalteau assay. Briefly, 200 µL extract was added to 1,000 µL of 10% Folin-Ciocalteau solution. Next, 800 µL of 7.5% sodium carbonate (Na_2CO_3) was added and incubated for 15 min in the dark at RT. Finally, the absorbance was measured by a UV-visible spectrophotometer (Mecasys Optizen POP; Metertech) at 765 nm. Total phenolic content was calculated and presented as mg gallic acid equivalent (GAE)/g extract.

Major phenolic constituent characterization of PPW by high-performance liquid chromatography (HPLC)

The amount of major phenolic compounds in 3 g of dried PPW was analyzed by high-performance liquid chromatography at the Science and Technology Service Center, Faculty of Science, Chiang Mai University (Chiangmai, Thailand). Briefly, gallic acid (Merck; Darmstadt, Germany), Catechin and quercetin (Sigma-Aldrich, St. Louis, MO, USA) were used as references for identification and quantification. PPW extracts were separated using the Agilent 1200 Series High-Performance Liquid Chromatography (Agilent, Santa Clara, CA, USA). For Gallic acid and Catechin analysis, the mobile phase was a binary solvent system consisting of absolute methanol and 0.05% Acetic acid. The Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 Micron) Column was used and the flow rate was 0.5 mL/min. The absorption wavelength of Gallic acid and Catechin was recorded at 272 and 280 nm, respectively. For quercetin analysis, the mobile phase was a binary solvent system consisting of methanol: 2% acetic acid. The Poroshell HPH C18 (4.6 × 150 mm, 4 Micron) Colum was used and the absorption wavelength was recorded at 366 nm. The major phenolic compounds were detected by comparing them to the corresponding peak of each standard and the concentration of each compound was quantified using the peak area of the reference compound.

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

PPW extract was assessed for free radical scavenging activity using the DPPH radical assay (Fluka, Hannover, Germany). Briefly, PPW extracts were added to 150 mM of DPPH solution and mixed for 10 s. Then, the mixture was measured using a UV-visible spectrophotometer at 765 nm (Mecasys Optizen POP; Metertech). The percentage of DPPH activity was determined with this formula: % DPPH activity = $\{(A_0 - A_t)/A_0\} \times 100$, where A_0 is the absorbance of the control reaction and A_t is the absorbance of the testing solution.

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Determination of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity

The PPW extract was determined for free radical scavenging activity by ABTS assay. Briefly, PPW extracts were added to 7 mM of ABTS solution (Fluka) and mixed for 10 s. Then, the reaction mixture was measured using a UV-visible spectrophotometer at 734 nm (Mecasys Optizen POP; Metertech). The percentage of ABTS was analyzed with this formula: % ABTS = $\{(A_0 - A_t)/A_0\} \times 100$, Where A_0 is the absorbance of the control reaction and A_t is the absorbance of the testing solution.

Determination of Ferric Reducing Antioxidant Power (FRAP)

The reducing power of the PPW extract was evaluated using the FRAP assay. Briefly, the PPW extract was mixed with FRAP solution and incubated at 37°C for 4 min. Then, the mixture was measured using a UV-visible spectrophotometer at 593 nm (Mecasys Optizen POP; Metertech). The different concentrations of iron sulfate heptahydrate ($FeSO_4.7H_2O$) were used for the standard solution and the ascorbic acid was used for the positive control. Data are presented as mg Fe(II)/g fraction.

Cell culture

RAW 264.7 macrophages were cultured in an incubator at 37°C and 5% carbon dioxide (CO_2) with Dulbecco's modified Eagle medium (Gibco; Invitrogen; Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1X Antibiotic-antimycotic solution (Gibco). Macrophages were pretreated with PPW extract for 1 h before stimulation with 1 ug/mL of lipopolysaccharide (LPS) (Sigma-Aldrich, Burlington, MA, USA) for 24 h.

Determination of PPW cytotoxicity

The PPW extract was assessed on cell viability by 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Briefly, 2×10^4 cells of RAW 264.7 in 200 µL were cultured in a 96-well plate and then treated with different concentrations of PPW extract for 24 and 48 h. After incubation, cells were washed and 100 µL of complete media containing 20 µL of MTS solution (G3580; Promega, Madison, WI, USA) was added to each well. Next, PPW-treated cells were incubated in an incubator at 37°C and 5% CO_2 for 3 h. Finally, the absorbance of each well was measured using a microplate reader at 490 nm (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA). The percentage of cell viability was calculated using untreated cells as the control.

Determination of ROS production

PPW extracts evaluated the ROS production using 2',7'-dichlorofluorescein-diacetate (DCF-DA; Sigma-Aldrich). Briefly, 2×10^4 cells of RAW 264.7 in 200 µL were pretreated with 10–400 ug/mL of PPW extract for 1 h and then stimulated with or without 1 µg/mL LPS at 37°C in an incubator with 5% CO_2 for 24 h. Next, cells were washed twice with complete media. Then, cells were washed and 100 µL of complete media containing 20 µM of DCF-DA was added and incubated at 37°C with 5% CO_2 for 25 min. Finally, the fluorescence signals were measured using a microplate reader (Varioskan Flash; Thermo Fisher Scientific) with excitation at 485 nm and emission at 535 nm. The percentage of ROS production was calculated using untreated cells as the control.

Determination of nitric oxide (NO) production

The 2×10^4 cells of RAW 264.7 macrophages were pretreated with 10–400 ug/mL of PPW extract for 1 h and then stimulated with or without 1 µg/mL LPS at 37°C in an incubator with 5% CO_2 for 24 h. The NO production was examined using Griess reagents. Briefly, Griess

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reagents were prepared by mixing a 1:1 ratio of 0.1% NED (Sigma-Aldrich) in diH_2O and 1% of sulphanilamide (Sigma-Aldrich) in 5% phosphoric acid (Sigma-Aldrich). Also, 100 μL of supernatants were collected and incubated with 100 μL of Griess reagents for 10 min in the dark at RT. Finally, the signals were measured using a microplate reader (Varioskan Flash; Thermo Fisher Scientific) at 540 nm. The percentage of NO levels is calculated using untreated cells as the control.

Measurement of pro-inflammatory cytokines secretion

The pro-inflammatory cytokines of tumor necrosis factor-α (TNF-α), interleukins (IL)-6, IL-1β, interferon (IFN)-γ (the MAX Deluxe ELISA Kits; BioLegend, San Diego, CA, USA) and prostaglandin E2 (PGE2) (Abbkine, Wuhan, China) were measured according to the manufacturer's protocols. Briefly, the diluted capture antibody was coated to the enzyme-linked immunosorbent assay plates overnight at 4°C. After blocking, 1:20 dilutions of TNF-α, IL-6, IL-1β, and undiluted IFN-γ of collected supernatant were added and incubated for 2 h at RT with a shaker. Next, 100 μL of detection antibody was added to the plate and incubated for 1 h. After incubation, HRP solution was added for 30 min at RT. The plates were washed and developed with substrate solution for 15 min in the dark. Finally, 2N H_2SO_4 was added. The plates were measured using a microplate reader (Varioskan Flash; Thermo Fisher Scientific) at 450 nm. The concentration of cytokines is calculated using a standard calibration plot and presented as a pg/mL.

Gene expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocols, followed by RNA purification using RNeasy mini kit (QIAGEN, Hilden, Germany). Next, total RNA (1 μg) was used for cDNA synthesis using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The mRNA levels of interested genes were examined using the SensiFastTM SYBR Lo-ROX qPCR Kit (Sigma-Aldrich) with an Applied Biosystems 7500 Real-Time PCR machine (Applied Biosystems, Waltham, MA, USA) and the following thermal cycling conditions: 1 cycle of 95°C for 30 s followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The mRNA expression levels of the interested genes were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and assessed by the $2^{(-\Delta\Delta Ct)}$ method. The list of all primers used is provided.

IL-1β; F: 5'-AAAAAAGCCTCGTGCTGTCG-3', R: 5'-GTCGTTGCTTGGTTCTCCTTG-3'
IL-6; F: 5'-GTTCTCTGGGAAATCGTGGA-3', R: 5'-TGTACTCCAGGTAGCTATGG-3'
TNF-α; F: 5'-CGGGCAGGTCTACTTTGGAG-3', R: 5'-ACCCTGAGCCATAATCCCCT-3'
iNOS; F: 5'-GCCACCAACAATGGCAACAT-3', R: 5'-TCGATGCACAACTGGGTGAA-3'
COX-2; F: 5'-TGAGCACAGGATTTGACCAG-3', R: 5'-CCTTGAAGTGGGTCAGGATG-3'
Tlr9; F: 5'- GCTGTCAATGGCTCTCAGTTCC-3', R: 5'-CCTGCAACTGTGGTAGCTCACT-3'
Tlr7; F: 5'-GTGATGCTGTGTGGTTTGTCTGG-3', R: 5'-CCTTTGTGTGCTCCTGGACCTA-3'
Tlr8; F: 5'-AAGTGCTGGACCTGAGCCACAA-3', R: 5'-CCTCTGTGAGGGTGTAAATGCC-3'
MyD88; F: 5'-ACCTGTGTCTGGTCCATTGCCA-3', R: 5'-GCTGAGTGCAAACTTGGTCTGG-3'
Irf7; F: 5'-CCCAGACTGCCTGTGTAGACG-3', R: 5'-CCAGTCTCCAAACAGCACTCG-3'
Nf-Kb p65; F: 5'-TCCTGTTCGAGTCTCCATGCAG-3', R: 5'-GGTCTCATAGGTCCTTTTGCGC-3'
IFNγ; F: 5'-TTGCCAAGTTTGAGGTCAACAA-3', R: 5'-TGGTGGACCACTCGGATGA-3'
P22^{phox}; F: 5'-GCTCATCTGTCTGCTGGAGTATC-3', R: 5'-CGGACGTAGTAATTCCTGGTGAG-3'
Catalase; F: 5'-AAGACAATGTCACTCAGGTGCGGA-3', R: 5'-GGCAATGTTCTCACACAGGCGTTT-3'
Nrf2; F: 5'-CAGCATAGAGCAGGACATGGAG-3', R: 5'-GAACAGCGGTAGTATCAGCCAG-3'
Sod1; F: 5'-GGTGAACCAGTTGTGTTGTCAGG-3', R: 5'-ATGAGGTCCTGCACTGGTACAG-3'
GAPDH; F: 5'-CACTCACGGCAAATTCAACGGCAC-3', R: 5'-GACTCCACGACATACTCAGCAC-3'

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Determination of mtDNA copy number and transcription levels

The mitochondria were isolated with the Mitochondrial Isolation Kit (Abcam, Cat.: AB288084), following the manufacturer's instructions. The supernatants were collected without mitochondria. Genomic DNA from the supernatants was isolated using the PrimeWay Genomic DNA isolation kit (Biodesign) following the manufacturer's instructions. Next, 10 ng of cytosolic DNA was analyzed for the mMito and β2-microglobulin genes using the SensiFast™ SYBR Lo-ROX qPCR Kit (Sigma-Aldrich). The copy number was analyzed by mitochondrial DNA/nuclear DNA according to previous studies [25]. Total RNA was extracted to quantify the mtDNA transcription level, as described above. Cytochrome c oxidase subunit 6 (COX6) and NADH-ubiquinone oxidoreductase chain 1 (ND1) were amplified to analyze the transcription level of mtDNA. GAPDH was used to normalize the level of mitochondrial transcription. The list of all primers used is provided.

mMito; F: 5'-CGTACACCCTCTAACCTAGAGAAGG-3', R: 5'-GGTTTTAAGTCTTACGCAATTTCC-3'
mβ2m; F: 5'-TTCTGGTGCTTGTCTCACTGA-3', R: 5'-CAGTATGTTCGGCTTCCCATTC-3'
ND1; F: 5'-CCACGCTTCCGTTACGATCA-3', R: 5'-TATGGTGGTACTCCCGCTGT-3'
Cox6b1; F: 5'-GAACTGTTGGCAGAACTACCTGG-3', R: 5'-ATGACACGGGACAGAGGGACTT-3'

Statistical analysis

All statistics in this study were conducted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Multiple groups were analyzed using a one-way variance analysis. Statistically significant results with $P < 0.05$ were considered.

RESULTS

Total bioactive contents of PPW

We first determined the bioactive compounds present in PPW extracts using the colorimetric method. We found the PPW extract to contain important bioactive constituents (**Table 1**) with a total phenolic content of 150.66 ± 7.91 mg of GAE/g and total flavonoid content of 102.01 ± 18.27 mg of catechin/g. In addition, the total polyphenolic content was evaluated for major constituents by HPLC (**Table 2**) and HPLC chromatograms are presented in **Fig. 1**.

Table 1. Bioactive compounds in PPW detected by colorimetric assay

Bioactive compound	Amount
Total phenolic content (mg GAE/g)	150.66 ± 7.91
Total flavonoid content (mg catechin/g)	102.01 ± 18.27

The total phenolic and total flavonoid contents of PPW extract were examined with the colorimetric method. Results are presented as mean ± SD over three replicates.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; GAE, gallic acid equivalent.

Table 2. The quantitative amount of major polyphenolic constituents in PPW

Major component	Amount (mg/kg of PPW)
Gallic acid	132
Quercetin	37.6
Catechin	88.2
Kaempferol	ND
Hydroquinin	ND

The total major content of PPW extract was determined with HPLC.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; ND, not detected; HPLC, high-performance liquid chromatography.

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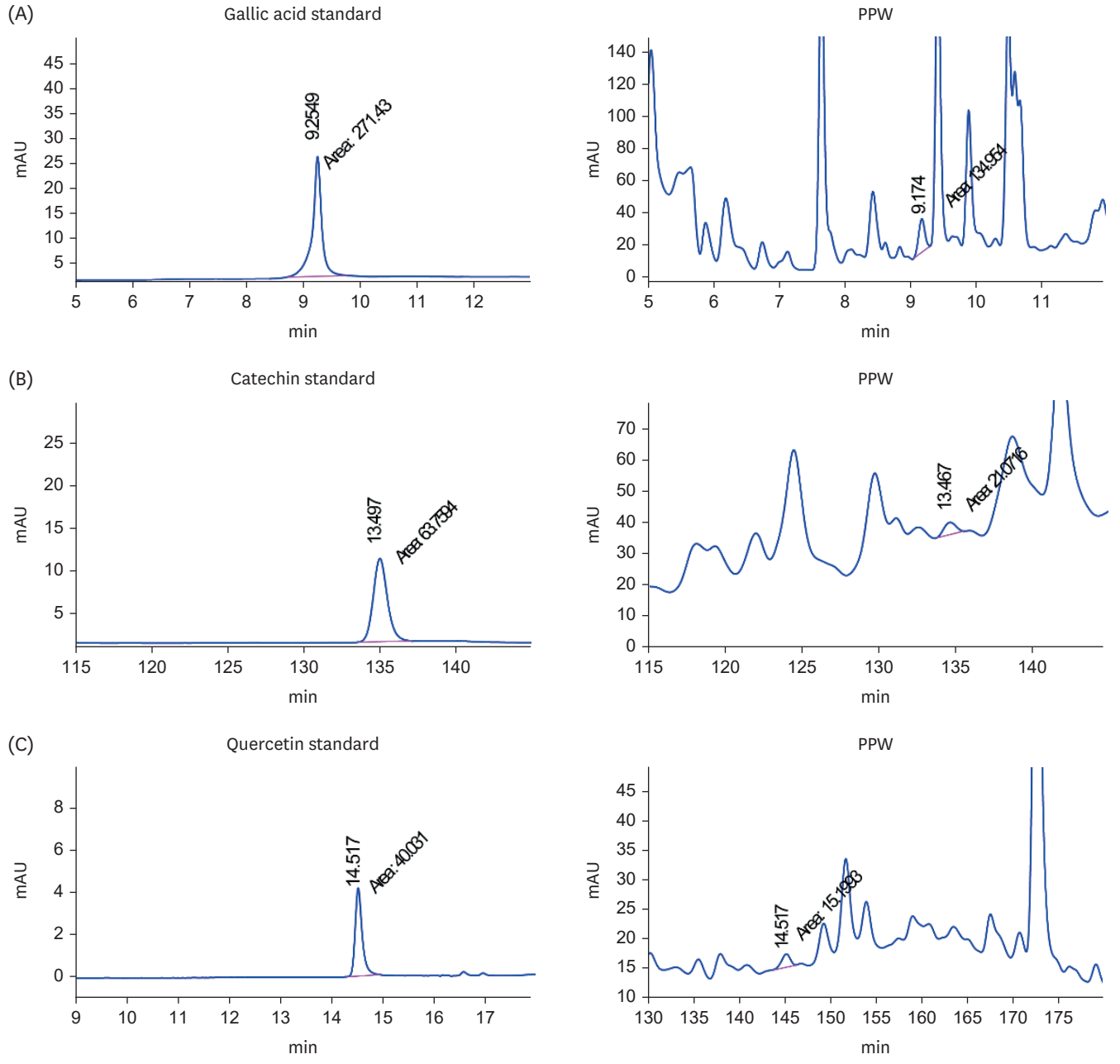

Fig. 1. HPLC chromatograms of polyphenolic constituents in PPW. (A) Gallic acid, (B) Catechin, and (C) Quercetin.
HPLC, high-performance liquid chromatography; PPW, *Pogonatherum paniceum* (Lamk) Hack extract.

Effect of PPW extract on antioxidant activity

DPPH and ABTS free radical scavenging activities were used to determine the antioxidant property of PPW extract. These data found the PPW extract to have a half-maximal inhibitory concentration (IC_{50}) against DPPH radicals of 73.87 ± 2.83 μg/mL, ABST radicals of 23.01 ± 0.78 μg/mL and a FRAP of 497.44 ± 6.96 mg Fe(II)/g fraction (**Table 3**). These results indicate that PPW extract has potent antioxidant activity that will be further explored in anti-inflammatory properties.

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Table 3. The antioxidant properties of PPW

Antioxidant activity	Amount
Radical scavenging assay	
ABTS IC_{50} (ug/mL)	23.01 ± 0.78
DPPH IC_{50} (ug/mL)	73.87 ± 2.83
Reducing power assay	
FRAP (mg Fe[II]/g fraction)	497.44 ± 6.96

The antioxidant properties of PPW extract were evaluated with DPPH and ABTS assays. Reducing power was investigated by the FRAP assay. Results are shown as mean ± SD across 3 replicates.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IC_{50}, half-maximal inhibitory concentration; FRAP, Ferric Reducing Antioxidant Power.

Effects of PPW extract on cell cytotoxicity

Next, the cytotoxicity effects of the PPW extract on macrophages were evaluated with an MTS assay. RAW 264.7 macrophages were treated with PPW extract for 24 and 48 h. We found cell viability to be unaffected by PPW extract at different doses between 10–800 μg/mL relative to the untreated cells (**Fig. 2A**).

PPW extract decreased the ROS and NO production related-genes

Further, ROS and NO production were investigated and the results show that 100–400 μg/mL of PPW extract significantly decreased ROS levels in activated macrophages (**Fig. 2B**). In addition, NO production in the culture medium was significantly reduced by 50–400 μg/mL extract (**Fig. 2C**). Also, cell viability after treatment with various concentrations of PPW extract with or without LPS stimulation did not differ from those of untreated cells (**Fig. 2D**). Moreover, we investigated the transcriptional levels of inflammatory-related genes and cytokines production at the different concentrations of PPW. Our results indicated that 400 μg/mL of PPW revealed the efficacy effects (data not shown). Therefore, the maximum dose of 400 μg/mL extract was observed in the subsequent experiment. Furthermore, we found that *p22*^{phox} and *iNOS* showed significantly lower mRNA transcription in activated macrophages treated with PPW relative to LPS-stimulated macrophages (**Fig. 2E and F**).

PPW extract increased the antioxidant enzyme-related gene expression

The data from this study indicated that the levels of *Catalase* mRNA expression were significantly higher in activated macrophages treated with PPW extract compared to LPS-induced macrophages alone ($P < 0.05$), as presented in **Fig. 2G**. However, no significant differences in the mRNA levels of *Nrf2* and *Sod2* were observed between these groups (**Fig. 2H and I**).

Effects of PPW extract on anti-inflammatory cytokine production

The elevated pro-inflammatory cytokine levels in the culture medium induced by LPS-stimulated macrophages were investigated. We found that mRNA transcription of *IL-6*, *TNF-α* and *IL-1β* was significantly higher after LPS stimulation but reduced in the presence of PPW extract (**Fig. 3A-C**). Furthermore, the LPS stimulation significantly increased the production of IL-6, TNF-α and IL-1β in the culture medium. On the other hand, increased cytokine production was attenuated in the presence of PPW extract (**Fig. 3D-F**). Also, 400 μg/mL of PPW extract significantly lowered *COX-2* mRNA transcription and PGE2 production in LPS-stimulated macrophages (**Fig. 3G and H**).

Effects of PPW extract on mitochondrial DNA leakage

To better understand whether the polyphenols present in the PPW extract affected mitochondrial stress. We evaluated the levels of mitochondrial DNA leakage in the cytosol.

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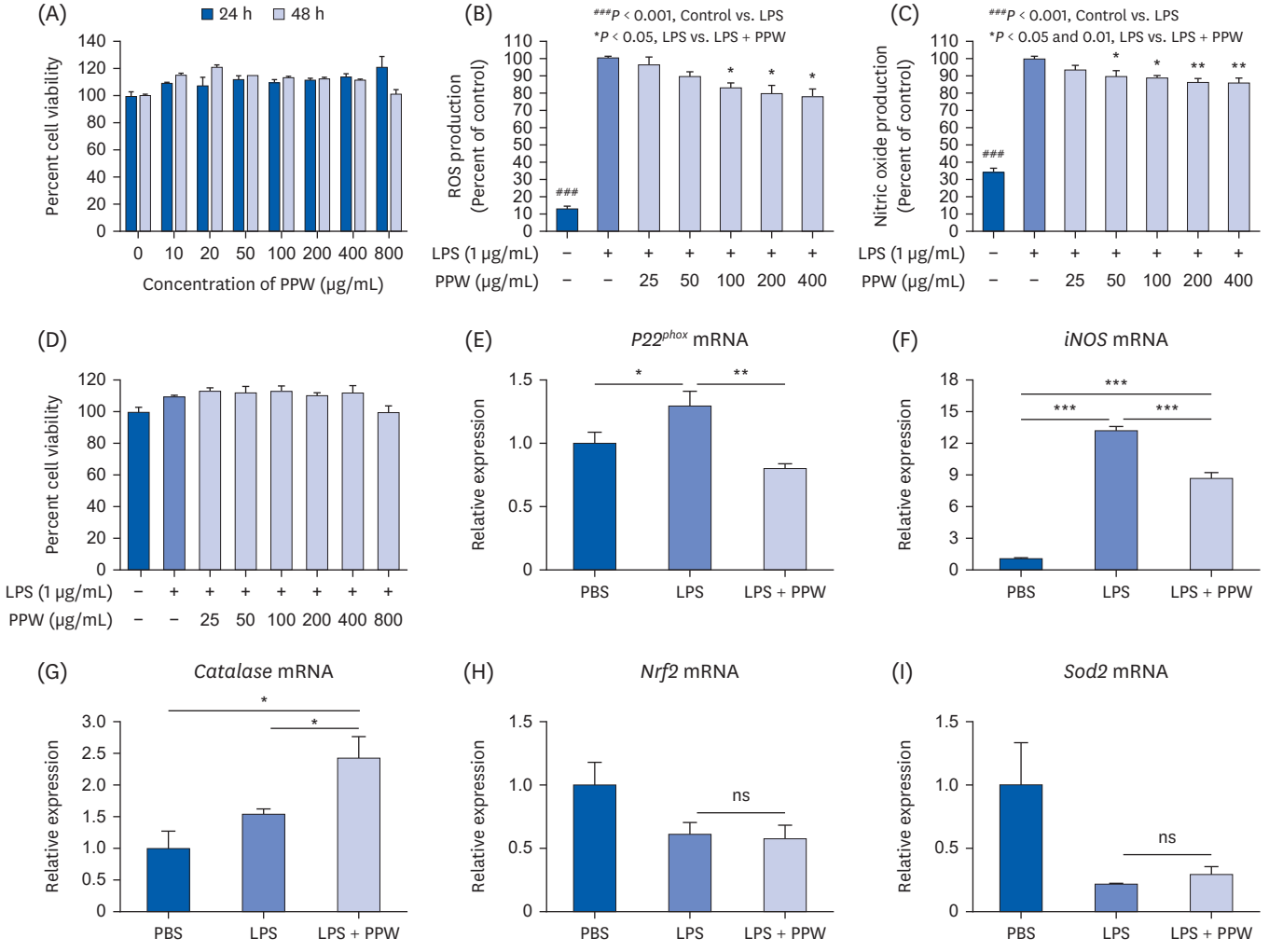

Fig. 2. Cytotoxic and antioxidant effects of PPW on macrophages. Macrophages were cultured and treated with different concentrations of PPW extract (10–800 ug/mL) for 24 and 48 h. (A and D) Cell viability was determined with the MTS assay using untreated cells as the control. Cells and supernatants were collected and analyzed for (B) ROS and (C) NO (n = 3 per group). mRNA expression of (E) *p22*^{phox}, (F) *iNOS*, (G) *Catalase*, (H) *Nrf2*, and (I) *Sod2* were investigated using qPCR (n = 3–6 per group). Data are presented as mean ± SEM. No significant differences are indicated between LPS-stimulated cells with or without PPW treatment.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; MTS, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium; ROS, reactive oxygen species; NO, nitric oxide; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; ns, not significant.
$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
$^{###}P < 0.001$, untreated cells compared with LPS-stimulated cells.

The results showed that LPS stimulation exhibited a significantly increased level of mtDNA in the cytoplasm relative to activated macrophages treated with PPW extract (**Fig. 4A**). However, the mtDNA transcription level of COX6 and ND1 showed a down-regulation of LPS stimulation and was significantly increased in PPW extract-treated cells (**Fig. 4B and C**). These data suggest that PPW extract can decrease mitochondrial stress, leading to a lower release of dsDNA to the cytoplasm.

Effect of PPW extract on TLR9 endosomal activation

Next, we explored whether the PPW extract can diminish mitochondrial DNA leaks and facilitate inflammatory responses in the LPS-stimulated macrophages. Our data showed that activated macrophages had significantly higher *Tlr9* mRNA expression relative to the activated cells treated with PPW extract (**Fig. 5A**), while *Tlr7* and *Tlr8* mRNA levels did not

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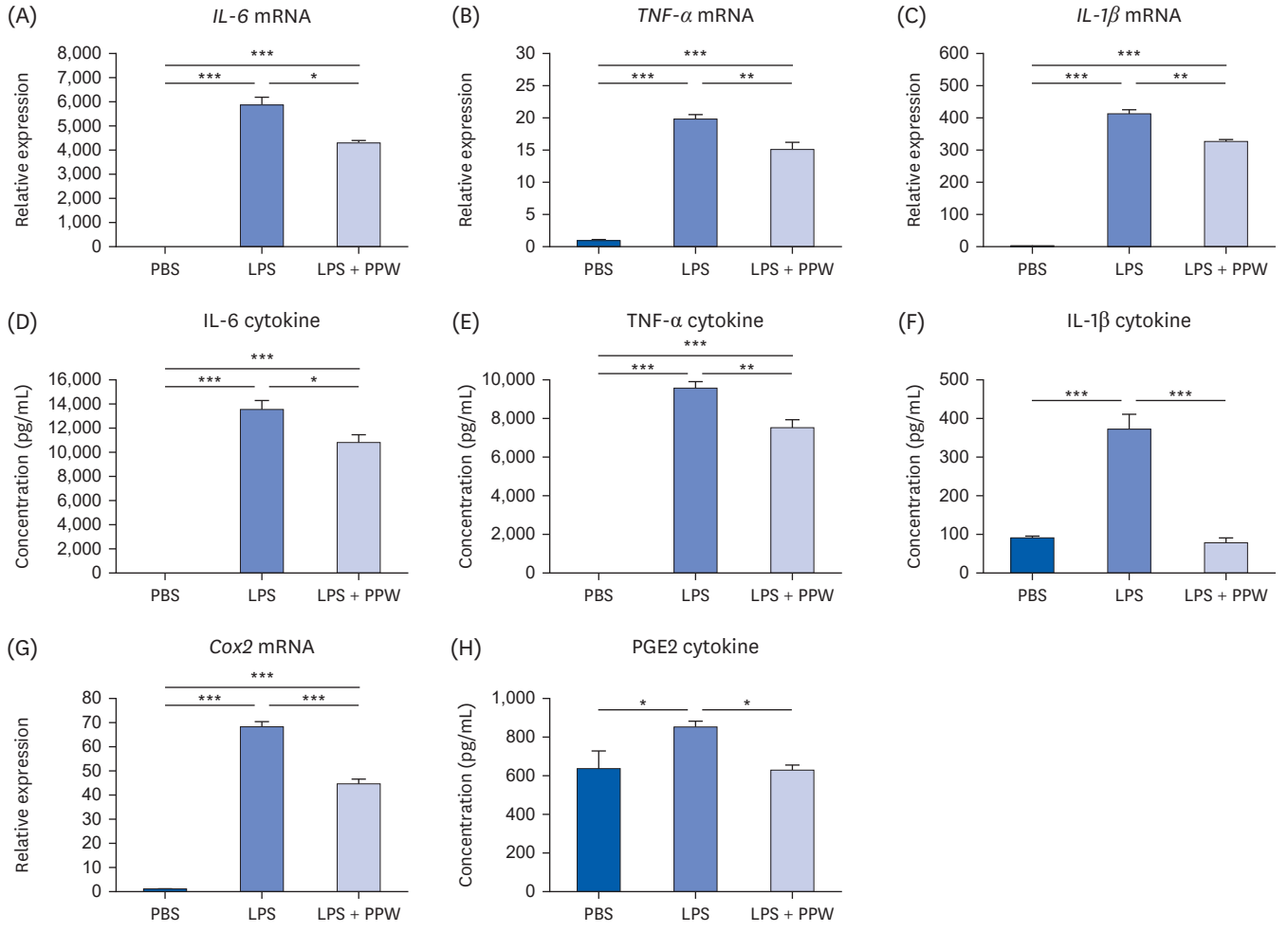

Fig. 3. Effects of PPW on anti-proinflammatory cytokine production. Cells were collected and examined the mRNA expression of (A) *IL-6*, (B) *TNF-α*, (C) *IL-1β*, and (G) *Cox2* by qPCR (n = 3–4 per group). The concentration of pro-inflammatory cytokines (D) IL-6, (E) TNF-α, (F) IL-1β, and (H) PGE2 (n = 3–4 per group) in the supernatant were examined by ELISA. Data are presented as mean ± SEM.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; IL, interleukin; TNF-α, tumor necrosis factor α; COX6, cytochrome c oxidase subunit 6; qPCR, quantitative polymerase chain reaction; PGE2, prostaglandin E2; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of mean.
$^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$.

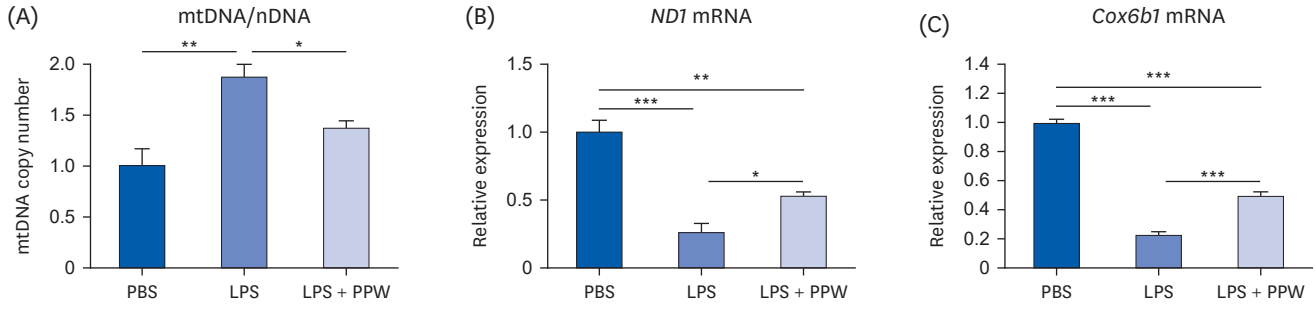

Fig. 4. Effects of PPW on mitochondrial DNA leakage. Cells were collected after the treatment of PPW with or without LPS and determined (A) the mtDNA leakage in cytoplasm (n = 4–6 per group). The mtDNA transcription level of (B) *Cox6* and (C) *ND1* was examined by qPCR (n = 3). Data are presented as mean ± SEM.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; LPS, lipopolysaccharide; mtDNA, mitochondrial DNA; COX6, cytochrome c oxidase subunit 6; ND1, NADH-ubiquinone oxidoreductase chain 1; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean.
$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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differ between these groups (**Fig. 5B and C**). We also detected the downstream Tlr9 activation in the endosome, including *MyD88*, *Irf7* and *NF-Kb p65* mRNA levels, showing significantly lower expression after the treatment of PPW in LPS stimulation relative to LPS-activated cells (**Fig. 5D-F**). Interestingly, PPW treatment significantly reduced *IFNγ* mRNA expression and IFN-γ cytokine production in the LPS-activated macrophages (**Fig. 5G and H**). These data confirm that PPW can reduce inflammatory responses by suppressing the *Tlr9* activation and signaling in LPS-induced mitochondrial DNA leakage.

DISCUSSION

The bioactive compounds obtained from medicinal plants are widely used against oxidative stress. Accumulating evidence indicated that elevated ROS production can cause extensive damage to tissues and biomolecules, including lipids, proteins and DNA [26], leading to

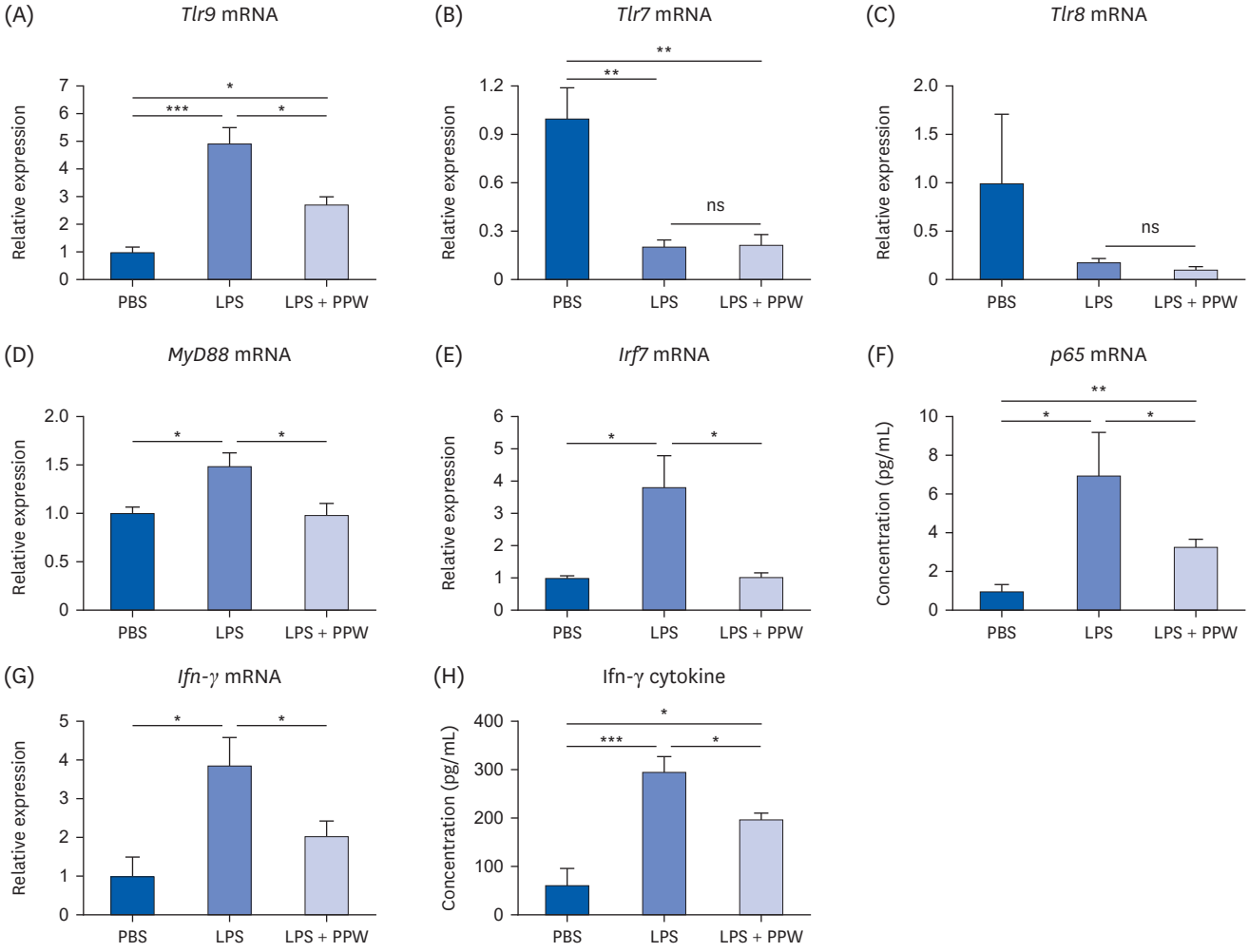

Fig. 5. The effect of PPW on endosome activation. Cells were collected and the mRNA transcription level of (A) *Tlr9*, (B) *Tlr7*, (C) *Tlr8*, (D) *MyD88*, (E) *Irf7*, (F) *Nf-Kb p65*, and (G) *Ifnγ* was examined by qPCR (n = 3–5 per group). (H) Supernatants were collected to measure the level of IFN-γ production (n = 3–5 per group). Data are presented as mean ± SEM. No significant differences are indicated between LPS-stimulated cells with or without PPW extract.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; qPCR, quantitative polymerase chain reaction; IFN, interferon; ns, not significant; SEM, standard error of mean; LPS, lipopolysaccharide.
$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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inflammation [3] and various diseases [27,28]. In this study, we identified and assessed bioactive compounds in the water extract from the perennial grass *Pogonatherum paniceum* (Lamk) Hack (PPW). We found that PPW extract contains high phenolic and flavonoid contents comparable to ethanol extract from several plants such as Sorghum (cereal) [29], *Saccharina japonica* [30], and *Aurea helianthus* leaf extract (Korean medicinal plant) [31].

Phenolic is an essential compound that performs as an antioxidant or free radical [32], which allows the stabilization of unpaired electrons of their structure [33]. Thus, we further explored the amount of major phenolic components with HPLC, containing gallic acid, quercetin and catechin, which all have reported antioxidant activity [34-36]. The *in vitro* studies found that PPW extracts have antioxidant activity against DPPH and ABST free radicals. Moreover, the reducing power assay (FRAP) shows the antioxidant abilities of this extract. These data also suggest that PPW extract has potent antioxidant effects similar to those of other medicinal plants [37-39]. Previous studies reported that decreased *iNOS* expression leads to lower NO production due to essential bioactive compounds such as gallic acid, catechin, rutin, quercetin and apigenin, which determine its effects [40-42]. Interestingly, several data suggest that gallic acid has the most significant activity against NO production [29,43] by inhibiting MAPK signaling [44]. In addition, NO can induce *COX-2* expression and enhance the levels of PGE2, a potent inflammatory mediator [45,46], resulting in the manifestation of inflammatory responses [47]. Our data suggested that inhibiting *iNOS* expression by PPW extract could be a potential mechanism for facilitating inflammatory responses.

Furthermore, we found that ROS production increased in LPS-stimulated macrophages, which can cause oxidative stress leading to the leaking of mitochondrial DNA to the cytoplasm [48] and promoting inflammatory responses through TLR9 activation [49]. The decreased *Tlr9* expression in PPW-treated cells was due to the reduction of mtDNA leakage. Accumulated evidence shows that catechin [50], gallic acid [51], and quercetin [52] facilitate mitochondrial stress via reduced ROS production by enhancing *catalase* expression. Moreover, these polyphenols were also found to inhibit cytokine-encoding genes, including IL-6, TNF-α, and IL-1β by suppressing TLR9/MyD88/NF-κB-dependent cytokines signaling pathway.

Our results showed that dsDNA from mitochondrial leakage interacts with TLR9, promoting the MyD88/IRF7-dependent interferon pathway and MyD88/NF-κB-dependent inflammatory cytokine signaling [53]. The decrease in cytokine levels may be due to the downregulated NF-κB activation [54,55] and p38 MAPK activation, which regulated the *IL-6*, *TNF-α*, and *IL-1β* expression [56] due to the polyphenols (quercetin, gallic acid, and catechin) present in PPW extract [44,57-59]. Also, TLR9 activation contributes to MyD88, which initiates signal transduction pathways that activate *Irf7*-mediated cytokine production in the presence of LPS [53,60,61]. This finding suggests that polyphenols in the PPW extract also inhibit TLR9/MyD88/IRF7-signaling, resulting in the lower expression of IFN-γ, as presented in **Fig. 6**.

This study highlights the antioxidant and anti-inflammatory activity of PPW extract and shows its polyphenolic contents, which have potential therapeutic uses. The data from this water extract could be a possible candidate for more advanced anti-inflammatory studies.

In conclusion, this study has established the antioxidant and anti-mtDNA mediated inflammation of PPW extract in LPS-induced macrophages. We found that the major polyphenolic constituents in the PPW extract have antioxidant activity against DDPH and

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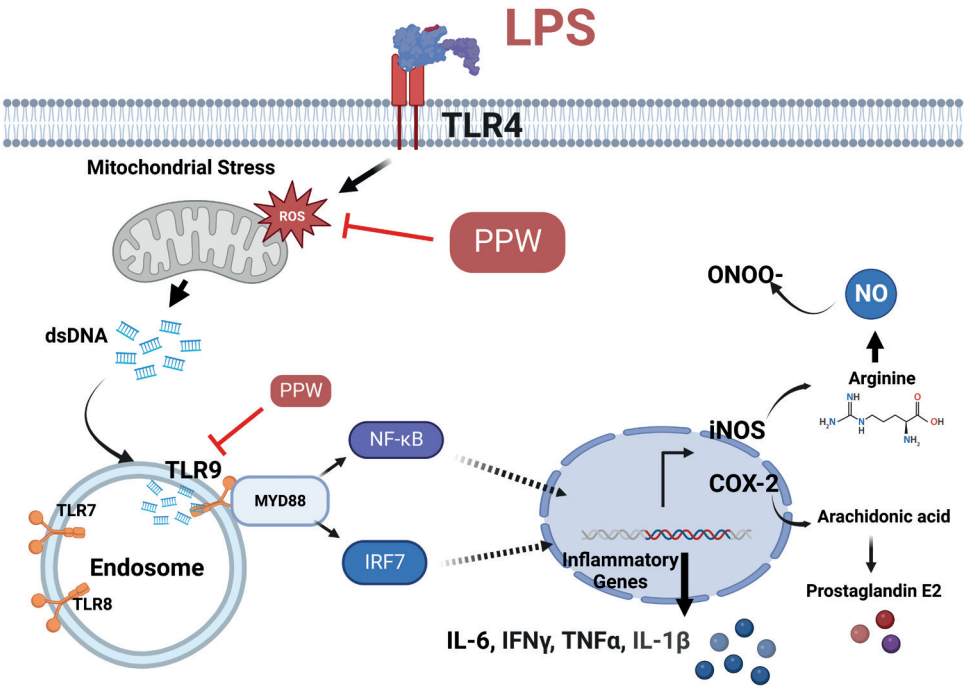

Fig. 6. Schematic summary of PPW on anti-inflammation in LPS-stimulated macrophages. PPW reduced the inflammatory response by decreasing the expression of *Tlr9* expression and signaling. This figure was created using Biorender.com.
PPW, *Pogonatherum paniceum* (Lamk) Hack extract; LPS, lipopolysaccharide; TLR, Toll-like receptor; ROS, reactive oxygen species; dsDNA, double stranded DNA; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; IRF, interferon regulatory factor; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; COX2, cytochrome c oxidase subunit 2; NO, nitric oxide.

ABTS free radicals. Therefore, these extracts improve the mitochondrial stress, leading to the decreased release of mtDNA to the cytoplasm. Moreover, the cytokine-encoding genes of IL-1β, IL-6, TNF-α, and IFN-γ in LPS-stimulated macrophages are reduced in the presence of PPW extract by attenuating the activation of TLR9 and signaling. This study highlighted the composition and biological effects of PPW extract and evaluated its potential mechanisms.

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