Anti-inflammatory effects of *Nypa fruticans* Wurmb via NF-κB and MAPK signaling pathways in macrophages

HYE JEONG PARK[1*], TAE WON JANG[2*], SO YEON HAN[1], SONG SOO OH[3], JUNG BOK LEE[4], SUNG MIN MYOUNG[5] and JAE HO PARK[2]

[1]Department of Medicinal Plant Science; [2]Department of Pharmaceutical Science, Jungwon University, Goesan-gun, Chungcheongbuk-do 28024; [3]Research Center, Kiposs Co., Ltd., Seoul 08584; [4]Kyochon Research & Innovation Center, Kyochon F&B, Jincheon-gun, Chungcheongbuk-do 27850; [5]Department of Public Health Administration, Jungwon University, Goesan-gun, Chungcheongbuk-do 28024, Republic of Korea

Received July 20, 2022; Accepted October 20, 2022

DOI: 10.3892/etm.2022.11690

Abstract. The inflammatory defense response of macrophages is a natural protective reaction in the immune system. Antioxidant and anti-inflammatory activities are closely related. In addition, the cell signaling pathway regulating inflammation is associated with MAPK and NF-κB signaling pathway phosphorylation. The present study aimed to evaluate whether the ethyl acetate fraction from *N. fruticans* (ENF) has a modulatory role in the MAPK signaling pathway and inhibition of the IκB/NF-κB signaling pathways, including translocation of NF-κB p65. Antioxidant and anti-inflammatory activities are closely related. In addition, the cell signaling pathway regulating inflammation is associated with MAPK and NF-κB signaling pathway phosphorylation. The results revealed that ENF exhibited antioxidant capacity, attenuated the cytokine levels and blocked nitric oxide production. ENF downregulated cyclooxygenase-2 and inducible nitric oxide synthase expression. We hypothesized that ENF treatment alleviated the various proinflammatory mediators via IκB phosphorylation and transcription of NF-κB compared with the untreated control. In conclusion, the present study demonstrated that the inhibitory effect of ENF treatment was attributed to the inhibition of MAPK and Akt/IκB/NF-κB signaling pathways.

Introduction

Nypa fruticans (*N. fruticans*) Wurmb is a mangrove plant (Araceae family). It is generally distributed throughout in the tropical regions of India, the Philippines, Malaysia, and some parts of Australia (1). Traditionally, the roots, stems, and leaves of *N. fruticans* have been used to remedy tuberculosis, sore throat, and liver diseases (2). *N. fruticans* contains polyphenols, flavonoids, protocatechuic acid, chlorogenic acid, and kaempferol (3,4). *N. fruticans* has antioxidant, antidiabetic, and hepatoprotective activities (4-7). Plants containing phenolic compounds exhibit anti-cancer and antioxidant activities in humans (8-11). Inflammatory responses serve in the immune defense system against stimuli such as infectious agent invasion or endotoxin exposure resulting in the retrieval of normal cell function and structure (12). Macrophages are activated by numerous ligands such as Fzd1, lipopolysaccharide (LPS), and RANKL (13-15). Activated macrophages release pro-inflammatory molecules [cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and cytokines (IL-1β, IL-6, and TNF-α)] (16). LPS propagates signaling cascades by stimulating major signaling pathways (17). The nuclear factor-kappa B (NF-κB) is found in numerous cell lines expressing cytokines and growth factors. NF-κB regulates a variety of target genes involved in cell survival, cell proliferation, and immune responses (18). Recent studies have shown that PI3K/Akt plays critical roles in various processes, including cell survival, cell cycle regulation, and NF-κB activation (19). MAPK, including ERK, JNK, and p38 mediate various cellular and biological processes related to inflammatory responses. MAPK phosphorylation activates the NF-κB signaling pathway (20). Impairment of these pathways could lead to diverse immunological diseases, including cancer and inflammation. Therefore, the MAPK, NF-κB, and PI3K/Akt signaling pathways are key targets for alleviating various inflammatory diseases. Aqueous extracts of *N. fruticans* have been previously reported (21). It has also been shown to have an inflammatory effect on sciatic neuropathy (22). We confirmed the effect of *N. fruticans* fractionated with ethyl acetate on the regulation of inflammatory factors. The present study aimed to verify the inhibitory effect of alleviating various pro-inflammatory mediators through regulation of the MAPK and Akt/IκB/NF-κB signaling pathways, including translocation of NF-κB p65.

Correspondence to: Professor Jae Ho Park, Department of Pharmaceutical Science, Jungwon University, 85 Munmu-ro, Goesan, Chungcheongbuk-do 28024, Republic of Korea
E-mail: parkjh@jwu.ac.kr

*Contributed equally

Key words: inflammation, macrophages, NF-κB, *Nypa fruticans* Wurmb, MAPK

Materials and methods

Chemicals and reagents. Dimethyl sulfoxide (DMSO), ethyl acetate, petroleum ether (PE), ethyl alcohol, and methyl alcohol were purchased from Merck. All chemical reagents were obtained from Sigma-Aldrich; Merck KGaA, unless otherwise stated. Antibodies were purchased from Cell Signaling Technology, Inc., Abcam and Santa Cruz Biotechnology, Inc.

Experimental materials extraction. Air-dried shoots of *N. fruticans* were purchased from Hantteus Yagcho (Seoul, Korea). The shoot extracts of *N. fruticans* (484.00 g) were immersed in 80% methanol (4.20 l) for 7 days using a sonicator (Hwashin Technology Co., Ltd.). The obtained extracts were then filtered by filter paper (Whatman™), evaporated under reduced pressure, and freeze-dried (N-1110S; Eyela). The powered *N. fruticans* was sequentially fractionated by organic solvents of increasing polarity (PE, chloroform, and ethyl acetate). The ethyl acetate fraction from *N. fruticans* (ENF, 0.38 g) was stored at 4˚C. ENF was diluted in DMSO and further used for experiments.

Analysis of total flavonoid contents (TFC). 100 μl of ENF (4 mg/ml) was mixed with 900 μl of distilled water, and followed by 1 ml of 10% $AlCl_3$. The absorbance of the reaction mixture was recorded at 420 nm using Xma-3000PC (UV/visible spectrophotometer; Human Corporation) after 30 min. TFC was determined by comparing it with the reference standard curve for rutin.

Antioxidant capacity evaluation. According to the method of Bondet and Van den Berg, radical scavenging activity was determined with some modifications (23,24). The 2,2-diphenyl-1-picryl hydrazyl (DPPH) solution (300 μM) was dissolved in ethyl alcohol. The ABTS solution containing 7.4 mM of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and potassium persulfate (2.6 mM) in distilled water was prepared for 24 h at ambient temperature. The DPPH or ABTS solution was adjusted to an absorbance value of 1.00 (515 nm or 732 nm, respectively). The sample (40 μl) was mixed with DPPH or ABTS solution (760 μl) and reacted for 30 min in the dark at ambient temperature. The data of radical scavenging activity was expressed as the decrease in the absorbance of DPPH or ABTS. The rate of decrease in the radicals was calculated using the formula as follows: $[1-(Abs_{Sample}-Abs_{Blank})/Abs_{Control}]$ x100 (%).

Here, Abs_{Sample}, Abs_{Blank} and $Abs_{Control}$ are the absorbance values of the sample, ethanol or distilled water and radicals, respectively. The standard compound, L-ascorbic acid, was used at the same concentration. Then, a curve was constructed, and the equation of the curve was used to calculate the half-maximal inhibitory concentration (IC_{50}, μg/ml).

Cell culture. Macrophages (ATCC® CRL-2278™; VA, USA) were used in this study. Cells were maintained in incubator (5% CO_2, 37˚C), and cultured with DMEM [1% penicillin/streptomycin and 10% FBS (Biowest)]. Then, 0.2% prophylactic plasmocin (InvivoGen) was used to suppress the proliferation of mycoplasma.

Cell viability. To estimate the cell proliferation assay, the cytotoxic effects of ENF treatment on macrophages were assessed by the CellTiter 96® AQueous One solution (Promega). The cells were incubated in each well of 96-well plate for 24 h. The ENF (25-400 μg/ml) treatment was done at the end of 24 h and the cells were reacted with the reagent for 2 h in an incubator (37˚C, 5% CO_2). The absorbance of supernatant was measured at 490 nm using a microplate reader (ELX808, BioTek).

Nitric oxide (NO) production. The cells ($4.0x10^5$ cells/well) were cultured in 6-well plates, and then treated with the appropriate concentration of ENF and 1 μg/ml of LPS (L6529, Sigma-Aldrich; Merck KGaA) for 24 h. Griess A reagent containing 1% sulfanilamide (w/v) in 5% phosphoric acid (v/v) and Griess B reagent containing 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (w/v) in distilled water were used. The supernatant of the culture medium was collected, and Griess A/B reagent (1:1) was mixed with it in equal volumes at room temperature for 10 min. To measure the NO production, absorbance was measured using a microplate reader at 540 nm.

Western blotting. Cells were seeded in 100 π dishes ($4x10^5$ cells/well). After 24 h, the cells were treated with ENF (25, 50, and 100 μg/ml) for 2 h and then induced with LPS (1 μg/ml) for 30 min or 24 h in macrophages. The cells were washed twice with ice-cold PBS. The cells were then lysed at 4˚C using a Radio immunoprecipitation assay (RIPA) buffer [Halt™ protease and phosphatase inhibitor cocktail, EDTA solution (Thermo Fisher Scientific, Inc.)]. Following the manufacturer's protocol, a Bradford assay (Bio-Rad) was employed to determine the protein concentration. After quantification, the protein was electrophoresed on a 12% polyacrylamide gel and electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). A 5% Bovine serum albumin (BSA, Biosesang) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) was used as a blocking agent at room temperature for 1 h. Subsequently, the membrane was incubated with primary antibodies (diluted in 3% BSA in TBS-T) at 4˚C for overnight. The primary antibodies (1:4,000) included an iNOS (D6B6S) monoclonal antibody (#13120), a phospho-NF-κB p65 monoclonal antibody (p-p65, #3033), an IκB-α (L35A5) monoclonal antibody (#4814), a phospho-IκB-α (Ser32) (14D4) monoclonal antibody (p-IκB-α,#2859), a phospho-Akt (Ser473) (D9E) monoclonal antibody (p-Akt, #4060), a SAPK/JNK polyclonal Antibody (#9252), a phospho-SAPK/JNK (Thr183/Tyr185) polyclonal antibody (p-JNK#9251), a p44/42 MAPK (ERK) (137F5) monoclonal antibody (#4695), a phospho-p44/42 MAPK (ERK) (Thr202/Tyr204) polyclonal antibody (p-ERK, #9101), a p38 MAPK (D13E1) monoclonal antibody (#8690), and a phospho-p38 MAPK (Thr180/Tyr182) polyclonal antibody (p-p38, #9211). Antibodies were purchased from Cell Signaling Technology. The anti-COX-2 polyclonal antibody (ab15919) and anti-NF-κB p65 polyclonal antibody (ab16502) were purchased from Abcam. The membrane was then washed thrice with TBS-T for 15 min. After washing, horse radish peroxidase (HRP)-labeled secondary antibodies, including anti-mouse IgG-HRP monoclonal antibody (18-8817-33, Rockland) and anti-rabbit IgG-HRP monoclonal antibody (18-8816-33) were incubated (1:10,000) for 1 h at room temperature. Protein bands were detected using an enhanced chemiluminescence (ECL) substrate solution (Bio-Rad) and visualized using the ChemiDoc imaging system (Bio-Rad).

Table I. Primer sequences.

A, RT-PCR

Gene	Primer sequence (5'-3')	Product size, bp
iNOS	F: AATGGCAACATCAGGTCGGCCATCACT	454
	R: GCTGTGTGTCACAGAAGTCTCGAACTC	
COX-2	F: GGAGAGACTATCAAGATAGT	861
	R: ATGGTCAGTAGACTTTTACA	
GAPDH	F: AACTTTGGCATTGTGGAAGG	130
	R: ATGCAGGGATGATGTTCTGG	
TNF-α	F: CACACTCAGATCATCTTCTC	198
	R: TTGAAGAGAACCTGGGAGTA	
IL-6	F: ATTACACATGTTCTCTGGGA	312
	R: TTTTACCTCTTGGTTGAAGA	
IL-1β	F: CAGGATGAGGACATGAGCAC	329
	R: CTCTGCAGACTCAAACTCCA	

B, RT-qPCR

Gene	Primer sequence (5'-3')	Product size, bp
iNOS	F: TGGTGGTGACAAGCACATTT	119
	R: AAGGCCAAACACAGCATACC	
COX-2	F: AGAAGGAAATGGCTGCAGAA	108
	R: CCCCAAAGATAGCATCTGGA	
GAPDH	F: CCTCCAAGGAGTAAGAAACC	143
	R: CTAGGCCCCTCCTGTTATTA	

COX-2, cyclooxygenase-2; F, forward; iNOS, inducible nitric oxide synthase; R, reverse; RT, reverse transcription; qPCR, quantitative PCR.

Western blotting data were quantified using ImageJ version 1.52a (developed at the National Institutes of Health).

Reverse transcription-polymerase chain reaction (RT-PCR). Macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) for 24 h. The following day, the cells were treated with 25, 50, and 100 μg/ml of ENF for 2 h and then induced with LPS (1 μg/ml) for 6 h. The cells were washed with PBS and lysed using 350 μl RLT buffer. Total RNA was extracted from macrophages using NucleoSpin® RNA Plus (Macherey-Nagel), as described by the manufacturer and cDNA was synthesized from 1 μg of extracted RNA using ReverTra Ace-α (Toyobo). The synthesized cDNA was used as a template, including Quick Taq® HS DyeMix (Toyobo) and primers (Table I; COX-2, iNOS, IL-1β, TNF-α, IL-6, and GAPDH). PCR was performed using a T100 Thermal Cycler (Bio-Rad) and the PCR cycles were as follows: 25-35 cycles of 30 s at 94˚C (denaturation) and 30 s at each following T_m value of primers (annealing/extension). The mRNA levels of the target genes were normalized to the housekeeping gene (GAPDH). The band density was evaluated using ImageJ software.

Quantitative PCR (qPCR). For qPCR, the cDNA templates obtained from the RT-PCR were mixed with the Quanti Tect® SYBR-Green PCR kit (Qiagen) and all the primers. Primer sequences were designed using Primer 3 (Table I). All reactions were performed using Rotor-Gene Q series software (Qiagen) for qPCR analysis and the reaction conditions were as follows: 95˚C for 15 min (amplification), 94˚C for 15 sec (denaturation), 60˚C for 30 sec (annealing), and 72˚C for 30 sec (extension) with 40 cycles. Housekeeping gene (GAPDH) was used as a control for relative quantification of mRNA expression. The formula $2^{-\Delta\Delta Cq}$ was used to analyze the mRNA expression levels, where $\Delta\Delta Cq=(Cq_{target}-Cq_{GAPDH})_{sample}-(Cq_{target}-Cq_{GAPDH})_{control}$ (25).

Immunofluorescence. The cells were cultured on glass coverslips for 24 h and then treated with ENF for 2 h and later with LPS (1 μg/ml) for 30 min. After treatment, cells were fixed with 4% paraformaldehyde (Biosesang) dissolved in PBS for 15 min at ambient temperature. The cells were then washed with PBS for 3 min and blocked with 0.1% Triton X-100 at room temperature. Next, the cells were incubated with an anti-NF-κB p65 (1:1,000) and an anti-IκB-α (1:1,000) at 4˚C overnight. Subsequently, we used an anti-rabbit IgG (Alexa Fluor® 568, ab175471) and an anti-mouse IgG (Alexa Fluor® 488, ab150113) to stain the cells. The cells were incubated with DAPI (D1306, Invitrogen; Thermo Fisher Scientific, Inc.) at room

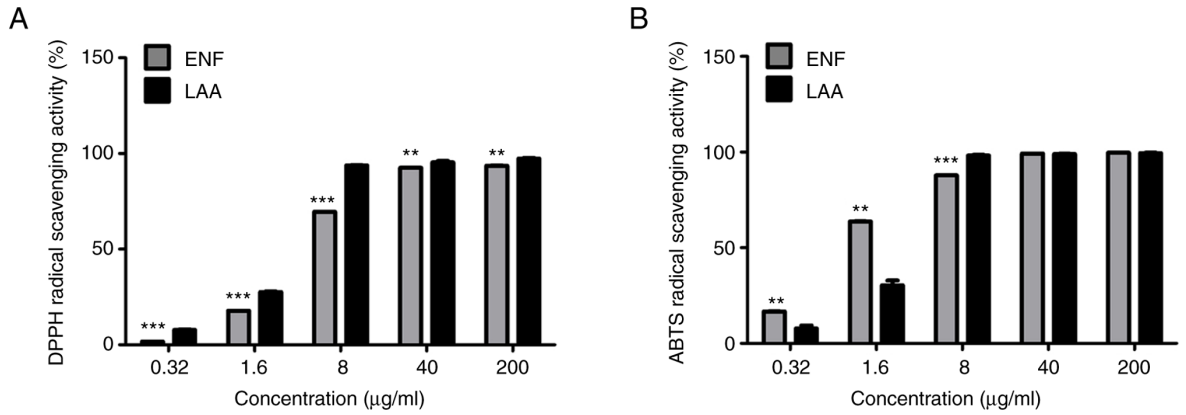

Figure 1. Antioxidant capacity of ENF. (A) DPPH radical scavenging activity of ENF. (B) ABTS radical scavenging activity of ENF. Values are expressed as the mean ± SD. **P<0.01 and ***P<0.001 vs. LAA at the same concentration. ABTS, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picryl hydrazyl; ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb; LAA, L-ascorbic acid.

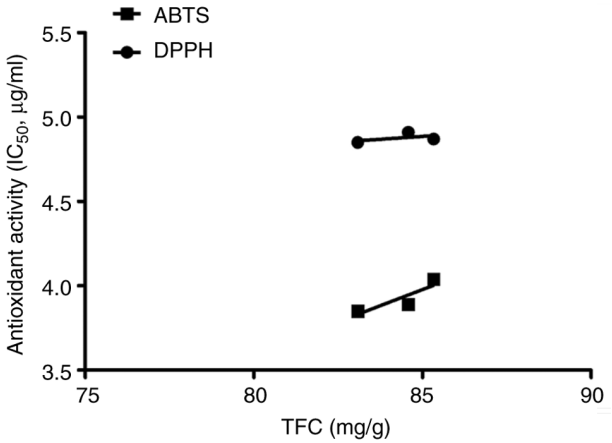

Figure 2. Pearson correlation curves for TFC and IC_{50} values of ABTS and DPPH radicals inENF. ABTS, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picryl hydrazyl; TFC, total flavonoid content; IC_{50}, half-maximal inhibitory concentration; ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb.

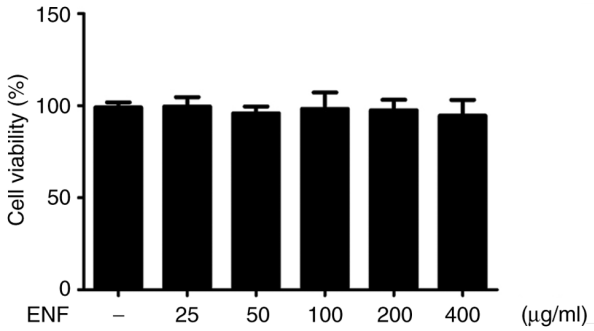

Figure 3. Cell viability analysis of ENF. Cell viability was presented as the percentage of each group compared with that in the control group (no ENF treatment; 100%). ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb.

temperature with light blocking. The cells were then washed at least five times with PBS. Finally, a drop of fluorescence mounting medium (S3023, Dako) was used prior to observation under a fluorescence at 400x magnification CKX53 microscope (Olympus) and images were obtained using a digital single-lens reflex camera (DS126271; Canon).

Statistical analysis. All experiments were repeated at least three times. Statistical analyses were verified using GraphPad Prism version 5.0 (GraphPad Software, Inc.) and data are presented as mean ± standard deviation. In the antioxidant capacity analysis of ENF, statistical comparisons were performed by unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. In other experiments, each data point was analyzed using one-way analysis of variance. The data were analyzed using the Bonferroni post hoc test.

Results

Antioxidant capacity of ENF. The results of the DPPH and ABTS radical scavenging are shown in Fig. 1, where it demonstrated the scavenging ability of ENF against DPPH and ABTS radicals in a dose-dependent manner. The DPPH radical scavenging activity of ENF showed maximum inhibition (93.60±0.13% at 200 μg/ml). Meanwhile, the ABTS radical scavenging activity of ENF showed maximum inhibition (99.67±0.12% at 200 μg/ml). Additionally, the TFC in ENF was 85.33 mg/g. The reference standard curve of the rutin was used for normalization (y=0.001x + 0.0433, R^2=0.9974). Pearson correlation coefficient statistics were analyzed to identify the relationship between total flavonoid content, ABTS radical scavenging activity, and DPPH radical scavenging activity (Fig. 2). A positive moderate correlation was found between the total flavonoid content and DPPH scavenging activity (r=0.506). There was a high positive correlation between the total flavonoid content and ABTS scavenging activity (r=0.865).

Effects of ENF on cell viability. To estimate the optimal ENF concentration not affecting the cell viability, macrophages were treated with ENF at various concentrations (25, 50, 100, 200, and 400 μg/ml). As shown in Fig. 3, concentrations ranging from 25-400 μg/ml showed no cytotoxicity as compared to that shown by the non-treated control (100%). Based on these studies (26,27), concentrations of 50 (95.83±3.73%) and 100 μg/ml (98.31±8.92%) were chosen for use in all experiments in this study.

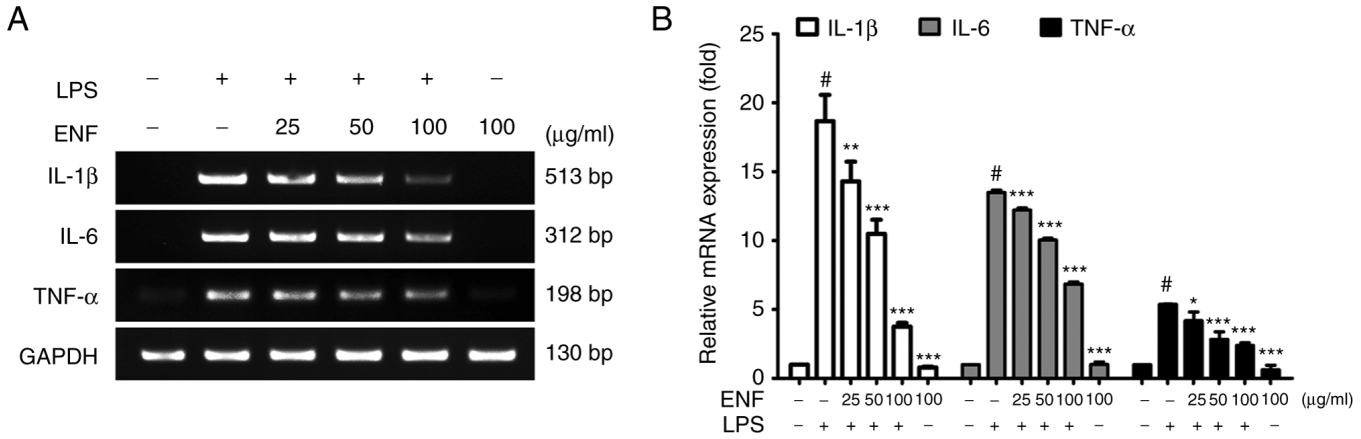

Figure 4. Inhibitory effect of ENF on cytokines. (A) Electrophoresis results of mRNA levels of cytokines. (B) Semi-quantification of IL-1β, IL-6, and TNF-α. Results were normalized to GAPDH expression. Values are presented as the mean ± SD. #P<0.001 vs. untreated group; *P<0.05, **P<0.01 and ***P<0.001 vs. LPS-induced group. ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb; LPS, lipopolysaccharide.

Effects of ENF on cytokines expression. Cytokines, including TNF-α, IL-6, and IL-1β are released to amplify the inflammatory response in macrophages (28). As presented in Fig. 4, the TNF-α mRNA levels were attenuated by ENF treatment (2.38±0.20-fold at 100 μg/ml) compared with that in the LPS-induced group (5.37±0.03-fold). The IL-6 mRNA level was attenuated by ENF treatment (6.85±0.16-fold at 100 μg/ml) compared with that in the LPS-induced group (13.48±0.18-fold). In addition, the IL-1β mRNA level was attenuated by ENF treatment (3.75±0.27-fold at 100 μg/ml) compared with that in the LPS-induced group (18.66±1.91-fold).

Effects of ENF on NO production, COX-2, and iNOS. We evaluated the modulatory role of ENF treatment on NO production in LPS-induced macrophages (Fig. 5A). Nitric oxide production was significantly increased by LPS (1509.36±71.53%) as compared with the non-treated control (100%). As presented in Fig. 5A, ENF treatment potently suppressed NO production at the concentration of 50 (875.44±28.07%) and 100 μg/ml (488.89±21.22%). As shown in Fig. 5C and D, the COX-2 and iNOS expression was increased by LPS (21.26±0.09-fold and 7.87±0.35-fold, respectively) at the cellular level. The COX-2 and iNOS expression was significantly suppressed (3.43±0.22-fold and 2.05±0.04-fold, respectively) by ENF treatment at 100 μg/ml. The COX-2 and iNOS mRNA levels correlated with protein expression by RT-PCR analysis (Fig. 5E). The mRNA level of COX-2 was notably attenuated by ENF treatment at 50 (4.70±0.50-fold) and 100 μg/ml (3.29±0.40-fold) as compared to the LPS-induced group (10.87±0.92-fold). The mRNA level of iNOS was attenuated through ENF treatment at 50 (6.74±0.13-fold) and 100 μg/ml (3.88±0.18-fold) as compared to the LPS-induced group (11.92±0.07-fold). Quantitative PCR (qPCR) was utilized to normalize mRNA levels and establish the results of RT-PCR analysis. The qPCR data followed a pattern similar to that of western blotting and RT-PCR analysis.

Effects of ENF on Akt/IκB/NF-κB signaling pathway. NF-κB is a transcription factor complex, that regulates the expression of proinflammatory mediators (29). As demonstrated in Fig. 6A, ENF treatment suppressed the phosphorylation of NF-κB p65 (0.99±0.03-fold at 100 μg/ml), without affecting the total amount of NF-κB p65. The p-IκB-α was increased by LPS (1.45±0.03-fold) at the cellular level and the p-IκB-α was significantly suppressed by ENF treatment (0.34±0.02-fold at 100 μg/ml). These results showed that ENF treatment downregulated the NF-κB signaling by suppressing p-IκB-α. Located in the nucleus, NF-κB p65 phosphorylation is a major step in the activation of NF-κB (30). To understand the molecular mechanism of NF-κB p65 translocation, we investigated the nuclear fluorescence of IκB-α and NF-κB p65, which are related to the expression of the NF-κB. As presented in Fig. 7, the NF-κB p65 was translocated from the cytoplasm to the nucleus as compared with the control group. Simultaneously, the fluorescence intensity of IκB-α in the nucleus was increased by ENF treatment of live cells. The increase in Akt phosphorylation results in the release of proinflammatory mediators, including cytokines, COX-2, and iNOS (31). As demonstrated in Fig. 6A, phosphorylation of Akt was increased by LPS (3.27±0.03-fold) at the cellular level. ENF treatment significantly suppressed the phosphorylation of Akt (1.39±0.05-fold at 100 μg/ml).

Effects of ENF on MAPK signaling pathway. MAPK (JNK, ERK, and p38) signaling pathway stimulates the NF-κB signaling pathway (32). To evaluate whether ENF exerts the modulatory role on phosphorylation of the MAPK, the JNK, ERK, and p38 expression was investigated by western blotting. As demonstrated in Fig. 6D, LPS enhanced the phosphorylation of JNK (7.38±0.54-fold), ERK (17.07±1.11-fold), and p38 (9.55±0.08-fold) at the cellular level. ENF treatment significantly suppressed p-JNK (1.67±0.05-fold), p-ERK (6.22±0.37-fold), and p-p38 (6.42±0.06-fold at 100 μg/ml) without affecting the total amount of JNK, ERK, and p38. A previous study reported that blocking the phosphorylation of MAPK pathways is associated with the regulation of proinflammatory mediators (33).

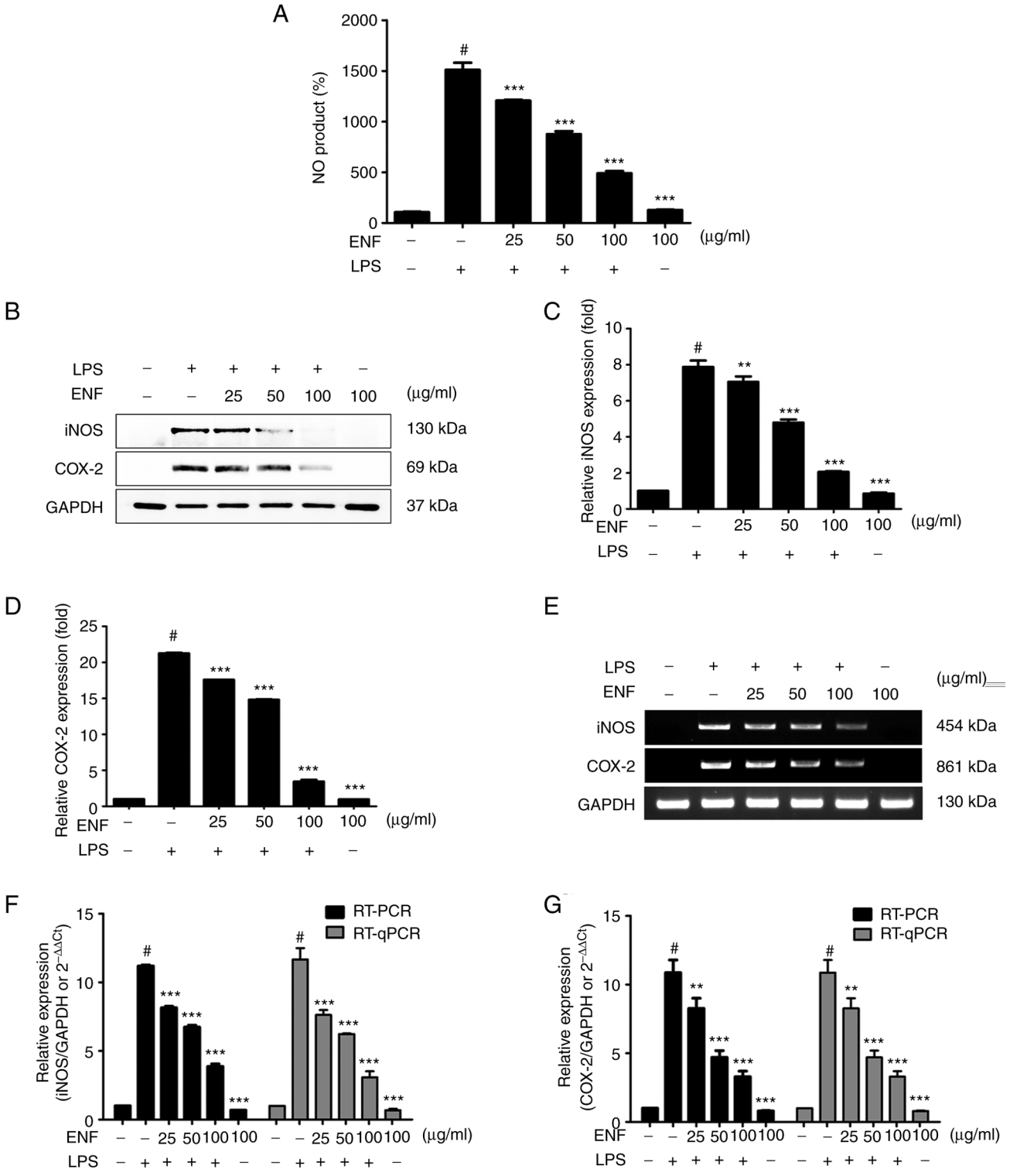

Figure 5. Inhibitory effects of ENF on macrophages. (A) Released NO in the culture medium was measured using a Griess reagent assay. (B) Western blotting results of iNOS and COX-2 expression. (C) iNOS expression. (D) COX-2 expression. (E) Electrophoresis results of mRNA levels of iNOS and COX-2. (F) mRNA levels of iNOS were examined using RT-qPCR analysis. (G) mRNA levels of COX-2 were examined using RT-qPCR analysis. Relative expression levels of iNOS and COX-2 are presented as a bar graph. Values are presented as the mean ± SD. $^{\#}$P<0.001 vs. untreated group; **P<0.01 and ***P<0.001 vs. LPS-induced group. ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; RT-qPCR, reverse transcription-quantitative PCR.

Discussion

Phytochemicals (alkaloids, terpenes, saponins, phenolics, and flavonoids) are naturally occurring compounds found in plants (34,35). They exert in the protection of human health, preventing various (inflammation, colorectal cancer, liver cancer, and DNA damage) diseases (36-39). Other plants belonging to the Araceae family contain phytochemicals such as sterols, saponins, phenols, and alkaloids (34,40,41). *N. fruticans* contains phytochemicals, such as chlorogenic

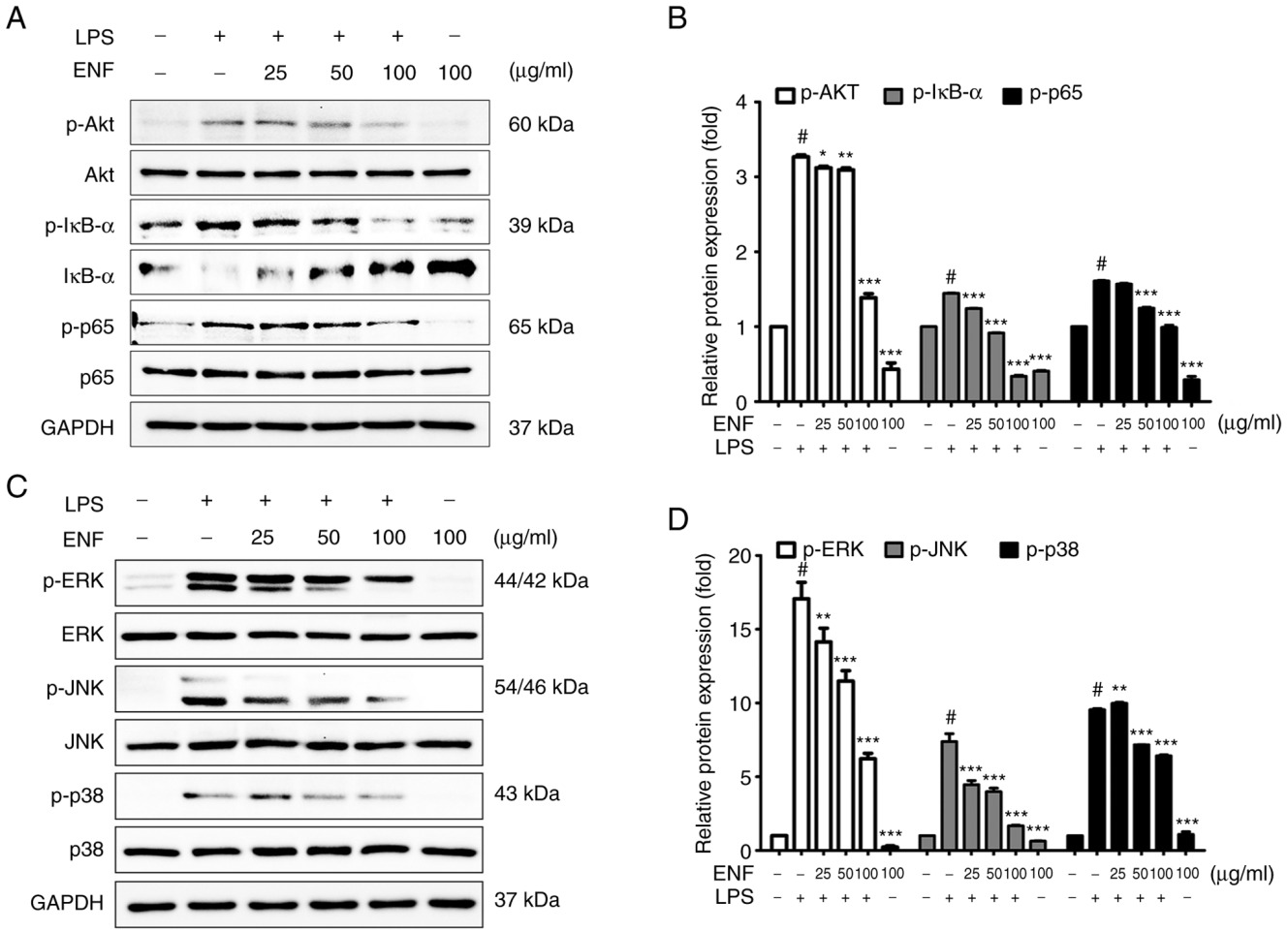

Figure 6. Inhibitory effect of ENF on Akt/IκB/NF-κB and MAPK signaling protein expression. (A) Western blotting results of protein phosphorylation levels of Akt, IκB-α, and NF-κB p65. (B) Phosphorylation levels of Akt, IκB-α, and NF-κB p65. (C) Western blotting results of protein phosphorylation levels of ERK, JNK, and p38. (D) Phosphorylation levels of ERK, JNK, and p38. Values are presented as the mean ± SD. #P<0.001 vs. untreated group; *P<0.05, **P<0.01, and ***P<0.001 vs. LPS-induced group. ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb; LPS, lipopolysaccharide; p-, phosphorylated.

acid, kaempferol, and protocatechuic acid (4,21,42), which have antioxidant, anticancer, and anti-inflammatory effects (43-45). There are studies related to the various bioactivities of *N. fruticans* such as antidiabetic, antioxidant, and anti-inflammatory effects (21,46,47). ENF showed a similar level of DPPH radical scavenging activity (IC_{50}, 3.93±0.10 μg/ml) compared to L-ascorbic acid (IC_{50}, 2.65±0.02 μg/ml). ENF scavenged ABTS radical (IC_{50}, 4.88±0.03 μg/ml) similar as compared to L-ascorbic acid (IC_{50}, 2.36±0.10 μg/ml). IC_{50} values for DPPH and ABTS radicals of ENF were not statistically significant when compared with L-ascorbic acid (P<0.001). These results can be inferred that the total flavonoid content in ENF contributes to its antioxidant capacity. Based on research on the relationship between flavonoid content and effects of anti-inflammatory (48) and capacity of free radicals in the inflammatory processes (49), it was confirmed that the anti-inflammatory effect of ENF was related to its antioxidant capacity. LPS-induced macrophages secrete cytokines, which are pro-inflammatory mediators that trigger inflammatory response (50). The transport of cytokines, including IL-6, IL-1β, and TNF-α was reduced by ENF treatment, which could lead to regulation of the cascade. Apparently, pro-inflammatory mediators are expressed in response to inflammation via cytokines (51,52). Since excessive NO production can lead to cytotoxicity (53), cancer (54), and autoimmune diseases (55), it is supported that blocking of iNOS expression and NO production can prevent inflammatory diseases. This study showed that ENF treatment suppressed NO production in LPS-induced macrophages. The suppression of COX-2 and iNOS expression by ENF showed a similar pattern to the inhibition of NO production, demonstrating that a decrease in the expression of iNOS by ENF suppresses NO production. At the basal level, NF-κB binds to IκB-α and is localized in the cytoplasm. An external stimulus, such as LPS induces phosphorylation of IκB-α, causing IκB degradation via ubiquitination of the NF-κB dimer. The released NF-κB p65 translocated into the nucleus and promotes the transcription of inflammatory mediators. We investigated the molecular mechanisms of IκB-α and NF-κB p65 in macrophages following ENF treatment. We confirmed that ENF treatment inhibited IκB-α phosphorylation, thereby attenuating the NF-κB p65 translocation to the nucleus. Immunofluorescence analysis showed that ENF treatment increased the fluorescence intensity of IκB-α, thereby increasing residual NF-κB p65 in the cytoplasm. The inhibition of IκB-α phosphorylation causes NF-κB p65 to

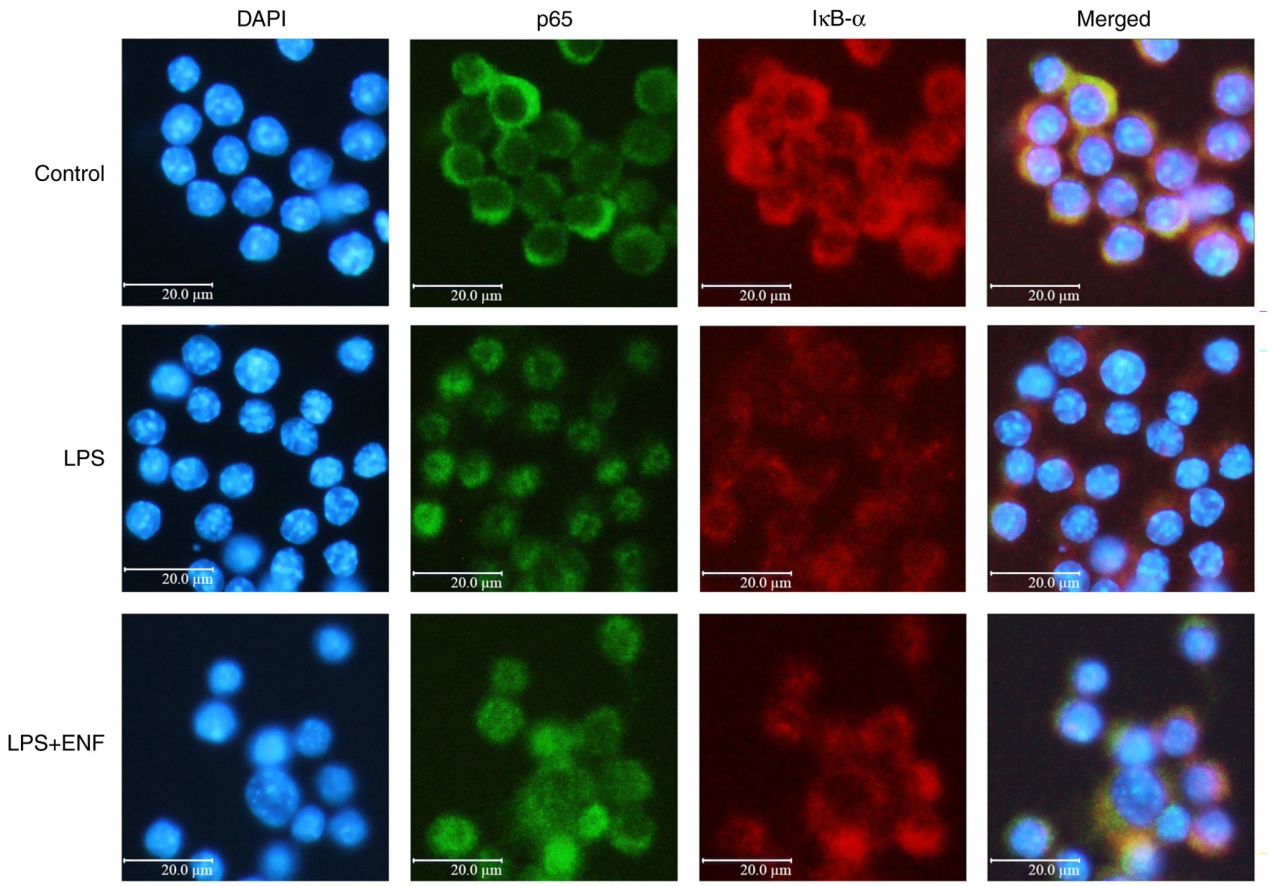

Figure 7. ENF regulates NF-κB p65 (green) and IκB-α (red) in macrophages. Fluorescence microscopy revealed the nuclear fluorescence of NF-κB p65 and IκB-α in macrophages treated for 30 min with LPS (1 μg/ml) + ENF (100 μg/ml). Scale bar, 20.0 μm. ENF, ethyl acetate fraction from *Nypa fruticans* Wurmb; LPS, lipopolysaccharide.

remain in the cytoplasm, and it is thought that ENF treatment regulates proinflammatory mediators by reducing the NF-κB signaling pathway. Akt is widely known as a downstream target of several cellular processes (56). The phosphorylation of Akt was inhibited by ENF treatment. Since Akt activates NF-κB by phosphorylating IκB kinase (IKK), inhibition of Akt phosphorylation through ENF treatment induces downstream NF-κB signaling pathway. Ligands activating cellular inflammatory responses induce phosphorylation of factors (JNK, ERK, and p38) in the MAPK signaling pathway in macrophages (57). ENF treatment suppressed the inflammatory cascade by alleviating the MAPK signaling pathway, which participate in NF-κB activation. In summary, ENF exhibits anti-inflammatory effects by modulating the nuclear translocation of NF-κB p65, which regulates the MAPK and Akt/IκB signaling pathways. The ethyl acetate fraction of *N. fruticans* can therefore be used as a functional material derived from natural products with the potential to regulate inflammatory responses.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

Not applicable.

Authors' contributions

HJP wrote the manuscript, performed the experiments and analyzed the data. HJP and SYH performed the experiments and interpreted the data. SOO and JBL participated in drafting the article. SSO, JBL and SMM were involved in interpretation of data. TWJ and JHP confirm the authenticity of all the raw data. JHP designed the experiments, wrote and edited the manuscript. HJP and TWJ made substantial contributions to the conception and acquisition of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Tamunaidu P and Saka S: Chemical characterization of various parts of nipa palm (*Nypa fruticans*). Ind Crops Prod 34: 1423-1428, 2011.
2. Bandaranayake W: Traditional and medicinal uses of mangroves. Mangroves and Salt Marshes 2: 133-148, 1998.
3. Hossain MF and Islam MA: Utilization of mangrove forest plant: Nipa palm (*Nypa fruticans* Wurmb.). Am J Agric Fores 3: 156-160, 2015.
4. Prasad N, Yang B, Kong KW, Khoo HE, Sun J, Azlan A, Ismail A and Romli ZB: Phytochemicals and antioxidant capacity from *Nypa fruticans* Wurmb. fruit. Evid Based Complement Alternat Med 2013: 154606, 2013.
5. Yusoff NA, Yam MF, Beh HK, Abdul Razak KN, Widyawati T, Mahmud R, Ahmad M and Asmawi MZ: Antidiabetic and antioxidant activities of *Nypa fruticans* Wurmb. vinegar sample from Malaysia. Asian Pac J Trop Med 8: 595-605, 2015.
6. Yusoff NA, Lim V, Al-Hindi B, Abdul Razak KN, Widyawati T, Anggraini DR, Ahmad M and Asmawi MZ: *Nypa fruticans* Wurmb. Vinegar's aqueous extract stimulates insulin secretion and exerts hepatoprotective effect on STZ-induced diabetic rats. Nutrients 9: 925, 2017.
7. Tsai SJ and Yin MC: Anti-glycative and anti-inflammatory effects of protocatechuic acid in brain of mice treated by D-galactose. Food Chem Toxicol 50: 3198-3205, 2012.
8. Ahn SK, Goo YM, Ko KH, Lee SJ, Moon YH, Lee SS, Kim JW and Lee SS: Study on the evaluation of nutritional values and antioxidant activities for herbal medicine by-products. J Agric Life Sci 48: 101-110, 2014.
9. Kim EY, Baik IH, Kim JH, Kim SR and Rhyu MR: Screening of the antioxidant activity of some medicinal plants. KOREAN J FOOD SCI. TECHNOL 36: 333-338, 2004.
10. Nirmala MJ, Samundeeswari A and Sankar PD: Natural plant resources in anti-cancer therapy-A review. Res Plant Biol 1: 01-14, 2011.
11. Wang Q, Kuang H, Su Y, Sun Y, Feng J, Guo R and Chan K: Naturally derived anti-inflammatory compounds from Chinese medicinal plants. J Ethnopharmacol 146: 9-39, 2013.
12. Lawrence T, Willoughby DA and Gilroy DW: Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2: 787-795, 2002.
13. Neumann J, Schaale K, Farhat K, Endermann T, Ulmer AJ, Ehlers S and Reiling N: Frizzled1 is a marker of inflammatory macrophages, and its ligand Wnt3a is involved in reprogramming Mycobacterium tuberculosis-infected macrophages. FASEB J 24: 4599-4612, 2010.
14. Sweet MJ and Hume DA: Endotoxin signal transduction in macrophages. J Leukoc Biol 60: 8-26, 1996.
15. Huang R, Wang X, Zhou Y and Xiao Y: RANKL-induced M1 macrophages are involved in bone formation. Bone Res 5: 17019, 2017.
16. So MS, Lee JS and Yi SY: Induction of nitric oxide and cytokines in macrophages by Codonopsis lanceolata. Korean J Food Sci Technol 36: 986-990, 2004.
17. Bjorkbacka H, Fitzgerald KA, Huet F, Li X, Gregory JA, Lee MA, Ordija CM, Dowley NE, Golenbock DT and Freeman MW: The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. Physiol Genomics 19: 319-330, 2004.
18. Kanarek N, London N, Schueler-Furman O and Ben-Neriah Y: Ubiquitination and degradation of the inhibitors of NF-kappaB. Cold Spring Harb Perspect Biol 2: a000166, 2010.
19. Rajaram MV, Ganesan LP, Parsa KV, Butchar JP, Gunn JS and Tridandapani S: Akt/Protein kinase B modulates macrophage inflammatory response to Francisella infection and confers a survival advantage in mice. J Immunol 177: 6317-6324, 2006.
20. Haque MA, Jantan I and Harikrishnan H: Zerumbone suppresses the activation of inflammatory mediators in LPS-stimulated U937 macrophages through MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways. Int Immunopharmacol 55: 312-322, 2018.
21. Bae GS and Park SJ: The anti-inflammatory effect of *Nypa fruticans* Wurmb. fruit on lipopolysaccharide-induced inflammatory response on RAW 264.7 cells. Korea J Herbol 31: 79-84, 2016.
22. Kang MS and Hyun KY: Antinociceptive and anti-inflammatory effects of *Nypa fruticans* wurmb by suppressing TRPV1 in the sciatic neuropathies. Nutrients 12: 135, 2020.
23. Bondet V, Brand-Williams W and Berset C: Kinetics and mechanisms of antioxidant activity using the DPPH. free radical method. LWT-Food Sci Technol 30: 609-615, 1997.
24. van den Berg R, Haenen GRMM, van den Berg H and Bast A: Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem 66: 511-517, 1999.
25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
26. Jang TW and Park JH: Anti-Inflammatory Effects of Abeliophyllum distichum Nakai (Cultivar Okhwang 1) Callus through Inhibition of PI3K/Akt, NF-κB, and MAPK Signaling Pathways in Lipopolysaccharide-Induced Macrophages. Processes 9: 1071, 2021.
27. Kim EA, Kim SY, Ye BR, Kim J, Ko SC, Lee WW, Kim KN, Choi IW, Jung WK and Heo SJ: Anti-inflammatory effect of Apo-9'-fucoxanthinone via inhibition of MAPKs and NF-kB signaling pathway in LPS-stimulated RAW 264.7 macrophages and zebrafish model. Int Immunopharmacol 59: 339-346, 2018.
28. Adcock IM, Ford PA, Bhavsar P, Ahmad T and Chung KF: Steroid resistance in asthma: Mechanisms and treatment options. Curr Allergy Asthma Rep 8: 171-178, 2008.
29. Caban M, Chojnacka K, Owczarek K, Laskowska J, Fichna J, Podsedek A, Sosnowska D and Lewandowska U: Spent hops (Humulus lupulus L.) extract as modulator of the inflammatory response in lipopolysaccharide stimulated raw 264.7 macrophages. J Physiol Pharmacol: Apr 27, 2020 (Epub ahead of print).
30. Biswas SK and Lewis CE: NF-κB as a central regulator of macrophage function in tumors. J Leukoc Biol 88: 877-884, 2010.
31. Ngabire D, Seong Y, Patil MP, Niyonizigiye I, Seo YB and Kim GD: Anti-inflammatory effects of aster incisus through the inhibition of NF-κB, MAPK, and akt pathways in LPS-stimulated RAW 264.7 macrophages. Mediators Inflamm 2018: 4675204, 2018.
32. Schulze-Osthoff K, Ferrari D, Riehemann K and Wesselborg S: Regulation of NF-kappaB activation by MAP kinase cascades. Immunobiology 198: 35-49, 1997.
33. Kim NY, Cheong SH, Lee KJ, Sok DE and Kim MR: Anti-inflammatory effects of Ribes diacanthum Pall mediated via regulation of Nrf2/HO-1 and NF-κB signaling pathways in LPS-stimulated RAW 264.7 macrophages and a TPA-induced dermatitis animal model. Antioxidants (Basel) 9: 622, 2020.
34. El-Desouky SK, Kim KH, Ryu SY, Eweas AF, Gamal-Eldeen AM and Kim YK: A new pyrrole alkaloid isolated fromArum palaestinum Boiss. and its biological activities. Arch Pharm Res 30: 927-931, 2007.
35. Koes RE, Quattrocchio F and Mol JNM: The flavonoid biosynthetic pathway in plants: Function and evolution. BioEssays 16: 123-132, 1994.
36. Leiherer A, Mündlein A and Drexel H: Phytochemicals and their impact on adipose tissue inflammation and diabetes. Vascul Pharmacol 58: 3-20, 2013.
37. Johnson IT: Phytochemicals and cancer. Proc Nutr Soc 66: 207-215, 2007.
38. Rajendran P, Ho E, Williams DE and Dashwood RH: Dietary phytochemicals, HDAC inhibition, and DNA damage/repair defects in cancer cells. Clin Epigenetics 3: 4, 2011.
39. Nishino H, Satomi Y, Tokuda H and Masuda M: Cancer control by phytochemicals. Curr Pharm Des 13: 3394-3399, 2007.
40. Roshan R, Ahmed S and Hasan MM: Arisaema jacquemontii Blume (Araceae): A review of medicinal uses, phytochemistry and pharmacology. J Pharmacogn Phytochem 6: 429-432, 2017.
41. Dong H, Geng Y, Wang X, Song X, Wang X and Yu J: Chemical constituents from scindapsus officinalis (Roxb.) Schott. and their anti-inflammatory activities. Molecules 23: 2577, 2018.
42. Yusoff NA, Ahmad M, Al Hindi B, Widyawati T, Yam MF, Mahmud R, Razak KN and Asmawi MZ: Aqueous extract of *Nypa fruticans* Wurmb. vinegar alleviates postprandial hyperglycemia in normoglycemic rats. Nutrients 7: 7012-7026, 2015.
43. Sahoo S, Ghosh G, Das D and Nayak S: Phytochemical investigation and in vitro antioxidant activity of an indigenous medicinal plant Alpinia nigra BL Burtt. Asian Pac J Trop Biomed 3: 871-876, 2013.
44. Xu XH, Li T, Fong CM, Chen X, Chen XJ, Wang YT, Huang MQ and Lu JJ: Saponins from Chinese medicines as anticancer agents. Molecules 21: 1326, 2016.

45. Souto AL, Tavares JF, Da Silva MS, Diniz Mde F, de Athayde-Filho PF and Barbosa Filho JM: Anti-inflammatory activity of alkaloids: An update from 2000 to 2010. Molecules 16: 8515-8534, 2011.
46. Choi JH, Hwang JW, Lee SG, Heo SH and Kang H: Antioxidant effect of hot water extracts from 3 types Indonesia plants (Hibiscus petals, Moringa Oleifera gymnosperm, and Nipa Fruticans Wurmb). Journal of Naturopathy 10: 42-47, 2021.
47. Reza H, Haq WM, Das AK, Rahman S, Jahan R and Rahmatullah M: Anti-hyperglycemic and antinociceptive activity of methanol leaf and stem extract of *Nypa fruticans* Wurmb. Pak J Pharm Sci 24: 485-488, 2011.
48. Maleki SJ, Crespo JF and Cabanillas B: Anti-inflammatory effects of flavonoids. Food Chem 299: 125124, 2019.
49. Forman HJ and Zhang H: Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. Nat Rev Drug Discov 20: 689-709, 2021.
50. Dang Y, Mu Y, Wang K, Xu K, Yang J, Zhu Y and Luo B: Papaverine inhibits lipopolysaccharide-induced microglial activation by suppressing NF-κB signaling pathway. Drug Des Devel Ther 10: 851-859, 2016.
51. Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV and Woolf CJ: Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410: 471-475, 2001.
52. Zeilhofer HU and Brune K: Analgesic strategies beyond the inhibition of cyclooxygenases. Trends Pharmacol Sci 27: 467-474, 2006.
53. Kröncke KD, Fehsel K and Kolb-Bachofen V: Nitric oxide: Cytotoxicity versus cytoprotection-how, why, when, and where? Nitric Oxide 1: 107-120, 1997.
54. Hofseth LJ, Hussain SP, Wogan GN and Harris CC: Nitric oxide in cancer and chemoprevention. Free Radic Biol Med 34: 955-968, 2003.
55. Liew FY: Regulation of nitric oxide synthesis in infectious and autoimmune diseases. Immunol Lett 43: 95-98, 1994.
56. Grandage VL, Gale RE, Linch DC and Khwaja A: PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kB, MAPkinase and p53 pathways. Leukemia 19: 586-594, 2005.
57. Bode JG, Ehlting C and Häussinger D: The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. Cell Signal 24: 1185-1194, 2012.