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Original Article

Protective effect of Perilla leaf extract against ROS formation and inflammation induced by TNF-α in A549 human lung carcinoma cell line

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Abstract

Perilla frutescens is an herb traditionally used to treat chronic diseases owing to its antioxidant and anti-inflammatory properties. This study aimed to determine the antioxidant and anti-inflammatory effects of perilla leaf extract (PLE) in TNF-α-treated A549 human lung carcinoma cell line. *Perilla frutescens* leaves were extracted with 70% ethanol, and the phenolic, flavonoid, and rosmarinic acid contents were determined. Cytotoxicity of PLE was assessed using the MTT assay. The antioxidant activity and intracellular ROS production in TNF-α-treated A549 cells were evaluated. Furthermore, mRNA expression of pro-inflammatory mediators was assessed by RT-qPCR. These results showed that PLE contained phenolic acid, flavonoids, and rosmarinic acid and had no cytotoxicity. PLE exhibits antioxidant activity through a reduction in intracellular ROS production in TNF-α-treated A549 cells. Moreover, PLE showed significantly decreased mRNA expression of IL-1β, IL-8, TNF-α and COX-2. Thus, PLE plays an important role as an antioxidant and anti-inflammatory agent for the prevention of ROS formation and inflammation in A549 cells.

Keywords: *Perilla frutescens*, TNF-α, antioxidant, anti-inflammatory, A549 cancer cells

1. Introduction

Inflammation is a defense mechanism by which the body reacts to infection, toxic compounds, injury, or irritation, with the key features being pain, swelling, redness, and warmth. Inflammation plays an important role in tissue repair, remodeling, regeneration, and regulation of tissue homeostasis (Chen *et al.*, 2018). The pathophysiology of inflammation is a protective response associated with the immune system, including cells and chemicals. The inflammatory process correlates with the regulation of signaling pathways that control the levels of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and pro-inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Abdulkhaleq *et al.*, 2018; Kany, Vollrath, & Relja, 2019).

Although inflammation is a beneficial process for the body, if it leads to certain imbalances, this could induce disease. Inflammation is a cause of several chronic diseases, including arthritis, atherosclerosis, and cancer (Chen *et al.*, 2018). Inflammation predisposes an individual to the development of various types of cancer, including lung cancer, and it supports the initiation, promotion, and progression of carcinogenesis (Greten & Grivennikov, 2019). Many researchers have studied ways to overcome cancer-

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associated inflammation and have found that natural products with effective anti-inflammatory properties are candidate agents, owing to their few side effects and detectable advantages in the treatment of acute or chronic diseases (Zappavigna *et al.*, 2020).

Perilla frutescens is an edible plant that belongs to the mint family, Lamiaceae. Leaves and seeds of *P. frutescens* have been used as an indigenous food ingredient in Asian countries, including Thailand, Japan, Korea, and India (Ahmed, 2018; Yu *et al.*, 2017). Leaves of *P. frutescens* contains several bioactive compounds such as rosmarinic acid, apigenin, and luteolin, and have been traditionally used in medicines and in functional foods for years (Liu, Wan, Zhao, & Chen, 2013; Peng, Ye, & Kong, 2005). Previous studies found that perilla leaf extract (PLE) exhibited anti-aging, anti-allergic, and anti-cancer activities in human breast cancer and colon cancer (Khanaree, Pintha, Tantipaiboonwong, Suttajit, & Chewonarin, 2018; Mungmai, Preedalikit, Aunsri, & Amornlerdpison, 2020; Oh, Park, Ahn, Park, & Kim, 2011; Pintha *et al.*, 2018). The antioxidant and anti-inflammatory activities of PLE have been investigated in several cell types, such as in RAW264.7 macrophages (Huang, Lin, Chen, & Kao, 2014) and human neutrophils (Chen *et al.*, 2015). In addition, the anti-inflammation properties of PLE have been investigated in animal models (Kangwan, Pintha, Lekawanvijit, & Suttajit, 2019). However, to date, the mechanisms of action of the antioxidant and anti-inflammatory activities of PLE in A549 human lung carcinoma cell line have not been studied. The mechanisms of action in various cell types are different; therefore, we have to study the protective effects of PLE against TNF-α-induced reactive oxygen species (ROS) formation and inflammation in A549 cells for the development of improved targeted therapies.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals and reagents were of analytical grade, except for the chemicals used in RNA/DNA and high-performance liquid chromatography (HPLC) analysis, which were of molecular and HPLC grade, respectively. Folin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reagents were obtained from Merck KGaA (Darmstadt, Germany). Nucleospin RNA, ReverTra Ace qPCR RT master mix, 2X SensiFAST™ SYBR® Lo-ROX, primers, and TNF-α were purchased from Pacific Science (Bangkok, Thailand). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic solution were purchased from Gibco, Invitrogen™ (Grand Island, NY, USA).

2.2 Plant material

Perilla frutescens leaves were collected from a cultivation in Wiang-Sa district, Nan province, Thailand. The voucher specimen number QSBG-K2 was certified by Queen Sirikit Botanic Garden Herbarium, Chiang Mai, Thailand.

2.3 Preparation of perilla leaf extract

The dried leaves of *Perilla frutescens* were finely ground and then extracted with 70% ethanol with continuous stirring for 12 h and the extraction was performed twice. The mixture was filtered through Whatman No.1 filter paper. Next, the filtrate was evaporated in a rotary evaporator followed by freeze-drying to obtain a crude ethanolic extract of *Perilla frutescens* leaves (Pintha *et al.*, 2018).

2.4 Determination of total phenolic content

Total phenolic content (TPC) of PLE was evaluated using Folin–Ciocalteu assay (Pintha *et al.*, 2018). In brief, the PLE was mixed together with 10% Folin reagent and 7.5% sodium carbonate (Na_2CO_3). The reaction mixture was incubated in the dark for 30 min at room temperature. After 30 min, the absorbance was measured at 765 nm using a microplate reader. The TPC of PLE was calculated by comparing with the standard curve of gallic acid. The results are shown in milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract). All measurements were performed in triplicate.

2.5 Determination of total flavonoid content

Total flavonoid content (TFC) of PLE was determined by the aluminum chloride colorimetric method with slight modification (Pintha *et al.*, 2018). In brief, PLE was mixed together with deionized water and 5% sodium nitrite ($NaNO_2$). The reaction mixture was incubated for 5 min at room temperature. After 5 min, 10% aluminum chloride ($AlCl_3$) and sodium hydroxide (NaOH) were added, and the mixture was further incubated for 10 min. The absorbance was measured at 510 nm using a microplate reader. The TFC of PLE was calculated and compared with the standard curve of catechin. The results are shown in milligrams of catechin equivalent per gram of extract (mg CE/g extract). All measurements were performed in triplicate.

2.6 High performance liquid chromatography analysis of rosmarinic acid in perilla leaf extract

The rosmarinic acid content in PLE was determined using HPLC as previously described (Tipsuwan & Chaiwangyen, 2018). The rosmarinic acid was used as an authentic standard. PLE (10 mg/mL) was dissolved in methanol (HPLC grade) and further assessed by ODS-3-C18 column (4.6 mm x 250 mm, 5 µm particle diameter) (Agilent Technologies, Thailand). Trifluoroacetic acid in water (0.1%) and methanol were used as mobile phases A and B, respectively. The gradient program run was as follows: 0-35 min, 90-10% mobile phase A and 10-90% mobile phase B; 35-40 min, 10-90% mobile phase A and 90-10% mobile phase B. The flow rate of the mobile phase was set at 1 mL/min and the detection wavelengths were at 280 and 320 nm. The total rosmarinic acid content of PLE was calculated by comparison with the standard curve of rosmarinic acid. The results are shown in milligrams per gram of extract (mg/g extract). All measurements were performed in triplicate.

2.7 DPPH free radical scavenging activity assay

The free radical scavenging activity of PLE was measured using the DPPH radical scavenging assay with slight modifications (Tipsuwan & Chaiwangyen, 2018). PLE at various concentrations (20 µL) was added to 180 µL of 0.2 mM DPPH reagent in a 96-well plate. The reaction mixture was incubated in the dark for 15 min at room temperature. After 15 min, the absorbance of the mixture was measured at 517 nm using an ELISA plate reader. Methanol was used as a reagent blank of control and the mixture of methanol with various concentrations of the extract was used as a sample blank for the test. The experiments were repeated three times each at least in triplicate. The percentage of DPPH scavenging activity was compared with standard vitamin C and Trolox curves and calculated using the following formula:

$$\% \text{ DPPH scavenging activity} = \frac{((A_{control} - A_{blank}) - (A_{test} - A_{blank}))}{A_{control} - A_{blank}} \times 100$$

The antioxidant activity of PLE is presented as the concentration of the extract that scavenged free radicals by 50% (SC_{50}). The SC_{50} was calculated from the graph of % DPPH scavenging activity against PLE concentration.

2.8 ABTS radical scavenging activity assay

The free radical scavenging activity of PLE was measured using the ABTS radical scavenging assay with slight modifications (Tipsuwan & Chaiwangyen, 2018). PLE at various concentrations was added to ABTS solution. The reaction mixture was incubated in the dark for 6 min at room temperature. After 6 min, the absorbance of the mixture was measured at 734 nm using a spectrophotometer. Water was used as a reagent blank of control, and a mixture of water with various concentrations of PLE was used as a sample blank for the test. The experiments were repeated three times each at least in triplicate. The percentage of ABTS scavenging activity was compared with standard vitamin C and Trolox curves and calculated using the following formula:

$$\% \text{ ABTS scavenging activity} = \frac{((A_{control} - A_{blank}) - (A_{test} - A_{blank}))}{A_{control} - A_{blank}} \times 100$$

The antioxidant activity of PLE is presented as the concentration of the extract that scavenged free radicals by 50% (SC_{50}). The SC_{50} was calculated from the graph of % ABTS scavenging activity against PLE concentration.

2.9 Cell cultures

The human lung carcinoma cell line (A549) was obtained from the ATCC. The cells were maintained in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin. The cells were maintained at 37°C in a 5% CO_2 humidified incubator for further experiments.

2.10 Cell viability assay

The cell viability assay of PLE against A549 cells was determined using an MTT assay (Pintha *et al.*, 2018). In brief, A549 cells (1.5×10^3 cells/well) were seeded in a 96-well plate for 24 h. After 24 h, the cells were pretreated with various concentrations of PLE (0, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL) for 2 h and co-treated with TNF-α (100 ng/mL) for 24 h. Then, 5 mg/mL MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the treated cells in each well and incubated for 4 h at 37ºC and 5% CO_2. All solutions were removed and DMSO was added to the well. The absorbance was measured at 540/630 nm using an ELISA plate reader. All measurements were performed in triplicate.

2.11 Measurement of intracellular reactive oxygen species levels

Intracellular ROS levels were measured by the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay with slight modifications (Chumphukam *et al.*, 2018). A549 cells were incubated with DCFH-DA for 2 h. The excess DCFH-DA was removed by washing twice with phosphate buffered saline. Then the cells were co-treated with TNF-α (100 ng/mL) and PLE at various concentrations (0, 25, 50, 100, and 200 µg/mL). The mixture was incubated for 30 min at 37ºC and 5% CO_2. In the presence of ROS, the DCFH-DA was de-esterified to form a measurable fluorescent product. The fluorescence intensity presented in proportion to the intracellular ROS levels was quantified using a 96-well plate spectrofluorometer with an excitation wavelength of 480 nm and an emission wavelength of 530 nm. All measurements were performed in triplicate.

2.12 Measurement of pro-inflammatory mediator gene expression by RT-qPCR

To determine the effects of PLE on the gene expression of pro-inflammatory mediators including cytokines and enzymes in TNF-α-treated A549 cells, mRNA levels of IL-1β, IL-6, IL-8, TNF-α, COX-2, and iNOS were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

2.13 RNA extraction

A549 cells were incubated with various concentrations of PLE for 2 h and then induced with TNF-α for 24 h. In brief, the treated cells were extracted by adding lysis buffer with the β-mercaptoethanol mixture. Total RNA was separated and washed using a NucleoSpin RNA column (Macherey-nagel, Germany) and then eluted by RNase-free water. Total RNA quantity was measured using a NanoDrop spectrophotometer.

2.14 cDNA synthesis by reverse transcription

Total mRNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT master mix (TOYOBO, Japan), according to the manufacturer's instructions. In brief, reverse transcription reaction mixture contained total RNA (1 µg) and

5x RT master mix (2 µL) and the final volume was adjusted to 10 µL with molecular biology grade water. The mixture was incubated at 37˚C for 15 min, 50˚C for 5 min, and 98˚C for 5 min. The cDNA was further used as the template for qPCR.

2.15 Quantitative polymerase chain reaction (qPCR)

The reaction mixture was prepared by mixing 5 µL of cDNA, 10 µL of 2X SensiFAST™ SYBR® Lo-ROX (Bioline, UK), 3.4 µL of water, and 0.8 µL of 10 µM primers. The primer sequences of target genes used for qPCR are shown in Table 1. Expression levels were calculated by the comparative threshold (ΔΔCt) method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All measurements were performed in triplicate.

2.16 Statistical analysis

All experiments were performed in triplicate and are presented as mean ± standard deviation (SD). Statistical analysis was performed by Prism version 6.0 software using one-way ANOVA with Dunnett's test. Significant differences were called at $p < 0.05$.

3. Results and Discussion

3.1 Extract yield and phytochemical constituents of perilla leaf extract

The yield of 70% ethanolic extract of *Perilla frutescens* leaves was 9.88 %. The TPC was 231.94±3.87 mg GAE/g extract and the TFC was 297.52±3.46 mg CE/g extract. Rosmarinic acid in PLE was quantitatively determined by HPLC; the results are shown in Figure 1. It was found that PLE contained rosmarinic acid at 96.5±10.6 mg/g extract. Our results showed that PLE had high phenolic and flavonoid contents, especially of rosmarinic acid. Rosmarinic acid and other polyphenols as well as apigenin and luteolin—a main bioactive compound in PLE—are known to inhibit breast cancer cell invasion and migration by the reducing MMP-9 secretion and activity (Pintha *et al.*, 2018). The rosmarinic acid-enriched fraction of PLE has been shown to significantly decrease IL-1β, IL-6, TNF-α, COX-2, and PGE-2 mRNA expressions in an animal model (Kangwan *et al.*, 2019). Moreover, rosmarinic acid may act through several mechanisms, such as by exerting anti-inflammatory activity via the reduction of pro-inflammatory cytokines and by downregulation of nuclear factor-kappa B signaling pathway (Nadeem *et al.*, 2019).

3.2 Free radical scavenging activities of perilla leaf extract

Free radical scavenging activity of PLE was determined using the DPPH and ABTS radical scavenging assays. As shown in Figure 2, PLE inhibited DPPH and ABTS radicals in a dose-dependent manner. SC_{50} of DPPH free radical scavenging activity for PLE was 23.11±0.55 µg/mL. Comparison with reference antioxidants (SC_{50} of vitamin C= 8.48±0.17 µg/mL and SC_{50} of Trolox=10.24±0.20 µg/mL) indicated that PLE showed antioxidant activity. Likewise, SC_{50} of ABTS scavenging activity for PLE was 9.72±0.18 µg/mL to be compared with vitamin C (SC_{50} = 2.60± 0.03 µg/mL) and Trolox (SC_{50} = 3.47±0.03 µg/mL). A previous report indicated that PLE with high rosmarinic acid content inhibited the DPPH and ABTS radicals in a dose-dependent manner. Thus, PLE plays an important role in free radical scavenging and iron-chelating activities by interfering with the Fe^{2+}-ferrozine complex (Tipsuwan & Chaiwangyen, 2018). The antioxidant activity of PLE may be derived from rosmarinic acid and other polyphenols (Khanaree *et al.*, 2018; Phromnoi, Suttajit, & Saenjum, 2019). The findings have demonstrated that PLE exhibits antioxidative activity against oxidative stress-induced inflammation.

Table 1. Primer sequences for qPCR in A549 cells

Gene	Primer sequences
IL-1β	Forward : 5'-AAACAGATGAAGTGCTCCTTCCAGG-3'
	Reverse : 5'-TGGAGAACACCACTTGTTGC TCCA-3'
IL-6	Forward : 5'- ATGAACTC CTTCTCCACAAGC-3'
	Reverse : 5'- GTTTTCTGCCAGTGCCTCTTTG-3'
IL-8	Forward : 5'- AGATATTGCACGGGAGAA -3'
	Reverse : 5'- GAAATAAAGGAGAAACCA -3'
TNF-α	Forward : 5'-CCCAGGCAGTCAGATCATCTTC-3'
	Reverse : 5'-AGCTGCCCCTCAGCTTGA-3'
COX-2	Forward : 5'-CCCTTGGGTGTCAAAGGTAA-3'
	Reverse : 5'-GCCCTCGCTTATGATCTGTC -3'
iNOS	Forward: 5'-TCC GAG GCA AAC AGC ACA TTC A-3'
	Reverse: 5'-GGG TTG GGG GTG TGG TGA TGT-3'
GAPDH	Forward : 5'-GAAGGTGAAGGTCGAGTCA-3'
	Reverse : 5'-GCTCCTGGAAGATGGTGAT-3'

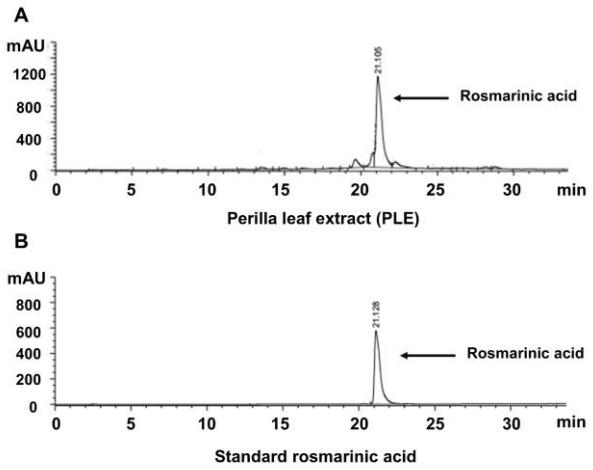

Figure 1. HPLC profiles of PLE (A), and standard rosmarinic acid (B). Rosmarinic acid content in PLE were determined using HPLC. Rosmarinic acid concentrations in PLE were calculated by comparison with the standard curve for rosmarinic acid.

3.3 Effects of perilla leaf extract on viability of TNF-α-treated A549 cells

The cytotoxicity of PLE in TNF-α-treated A549 cells was determined using an MTT assay. Figure 3 shows the cell viability of TNF-α-treated A549 cells cultured with different concentrations of PLE (0, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL). There was no significant effect on cell

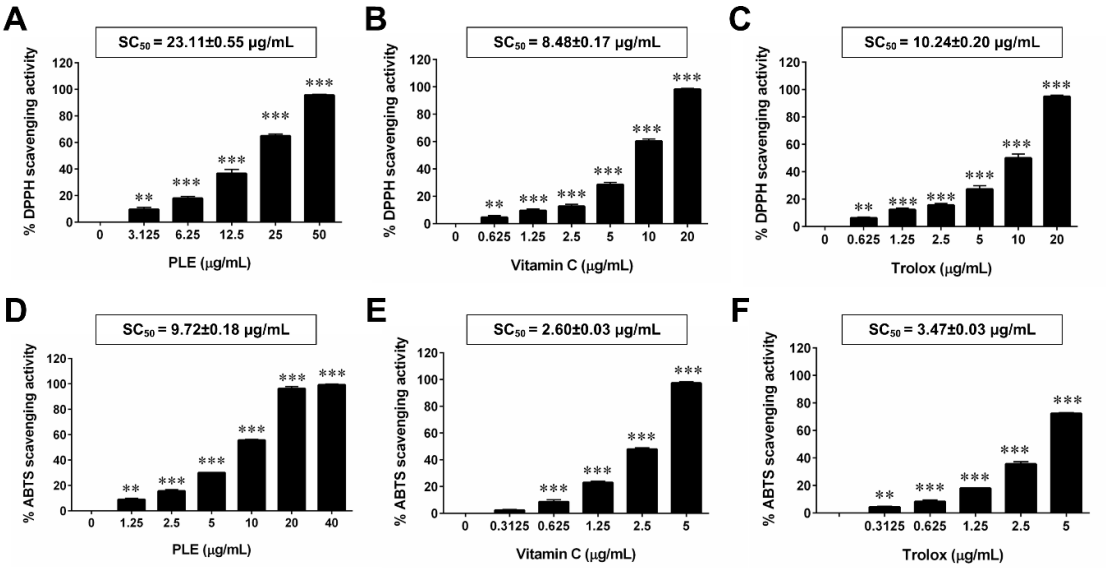

Figure 2. Effects of PLE on free radical scavenging activities were determined using DPPH and ABTS assays and comparing to standard vitamin C and Trolox. All measurements were performed in triplicate. The data are presented as mean ± SD., ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control.

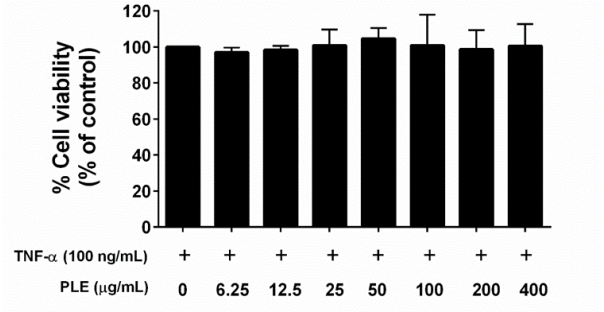

Figure 3. Effects of PLE on the viability of TNF-α-treated A549 cells were determined using MTT assay. TNF-α-treated A549 cells were incubated with various concentrations of PLE. All measurements were performed in triplicate. Data are presented as mean ± SD.

viability after treatment with PLE for 24 h. The 20% and 50% inhibitory concentrations (IC_{20} and IC_{50}, respectively) of PLE were >400 µg/mL. A non-toxic dose of PLE was used for further experiments. Similarly, in previous studies, no toxic effects of PLE were observed in various cell types, such as RAW 264.7 macrophages (Lee & Han, 2012). Thus, PLE can be used for biological studies in several cell types with low toxicity.

3.4 Effect of perilla leaf extract on intracellular ROS production

Oxidative stress is defined as an imbalance between production of free radicals and antioxidants. Reactive oxygen species (ROS) are involved in numerous of diseases, including chronic inflammation and cancers (El-Kenawi & Ruffell, 2017). TNF-α, a classical pro-inflammatory cytokine, plays an important role in immune homeostasis, inflammation, and host defense (Kany *et al.*, 2019). Additionally, TNF-α has been shown to increase ROS production in A549 lung cancer cells (Yang *et al.*, 2018). The effects of PLE on intracellular ROS production in TNF-α-treated A549 cells were determined by using the DCFH-DA assay. TNF-α-treated A549 cells were incubated with or without non-toxic concentrations of PLE (0, 25, 50, 100, and 200 µg/mL). As shown in Figure 4, TNF-α-treated cells had significantly higher intracellular ROS production when compared with non-treated cells ($p < 0.001$). TNF-α-induced cells treated with PLE at 100 and 200 µg/mL showed significantly lower intracellular ROS production than TNF-α-treated cells alone ($p < 0.05$). The antioxidative effects of PLE could reduce the ROS overproduction in TNF-α-treated A549 cells. As ROS are important mediators of inflammatory processes, neutralization of ROS by antioxidants and radical scavengers such as those in a medicinal plant extract may attenuate inflammation (Chatterjee, 2016). The results obtained from the present study demonstrate that PLE contains high levels of rosmarinic acid

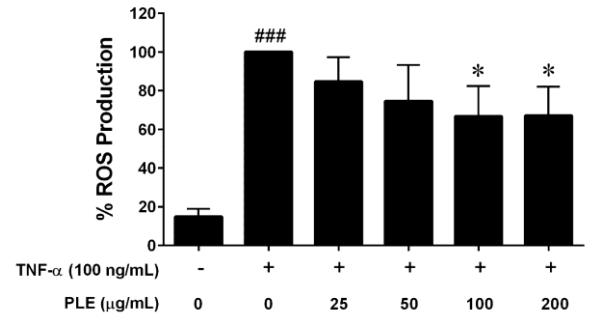

Figure 4. Effect of PLE on intracellular ROS production was determined using DCFH-DA assay. TNF-α-treated A549 cells were incubated with various concentrations of PLE. All measurements were performed in triplicate. The data are presented as mean ± SD., ### $p < 0.001$ compared to non-treated cells, *$p < 0.05$ compared to TNF-α-treated A549 cells alone.

and other polyphenols and neutralizes intracellular ROS production that causes cellular oxidative damage and inflammation.

3.5 Effect of perilla leaf extract on mRNA expression of pro-inflammatory cytokines

ROS are key mediators in the progression of inflammatory disorders (El-Kenawi & Ruffell, 2017). In lung cancer cells, ROS generation leads to overexpression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Yang *et al.*, 2018). The effects of PLE on IL-1β, IL-6, IL-8, and TNF-α mRNA expression in TNF-α-treated A549 cells were determined using RT-qPCR. As shown in Figure 5, TNF-α-treated A549 cells showed significantly higher mRNA expression of pro-inflammatory cytokines than non-treated cells ($p < 0.001$). Treatment with 200 µg/mL PLE significantly decreased the mRNA expression levels of IL-1β, IL-8, and TNF-α ($p < 0.01$ and $p < 0.001$) and slightly decreased mRNA expression of IL-6 (200 µg/mL). A previous study found that PLE could inhibit TNF-α, IL-1β and IL-6 production in LPS-stimulated RAW 264.7 cells (Lee & Han, 2012). Moreover, another study reported that PLE and rosmarinic acid could reduce IL-1β, IL-6, and TNF-α mRNA expressions in the nasal mucosa tissue of ovalbumin-sensitized mice (Oh *et al.*, 2011). Likewise, the rosmarinic acid-enriched fraction from perilla leaves reduced IL-1β, IL-6, and TNF-α mRNA expressions in rats with gastric ulcers induced by indomethacin (Kangwan *et al.*, 2019). The results obtained from the present study indicate that PLE reduces TNF-α-induced mRNA expression of pro-inflammatory cytokines such as IL-1β, IL-8, and TNF-α in A549 cells. This effect is associated with the inhibitory effect of PLE on ROS formation induced by TNF-α. The reduction in intracellular ROS levels results in the inhibition of IL-1β, IL-8, and TNF-α mRNA expression, which leads to the alleviation of the inflammatory process.

3.6 Effect of perilla leaf extract on COX-2 and iNOS mRNA expression

Pro-inflammatory enzymes such as COX-2 and iNOS play important roles in the development of chronic inflammatory diseases, such as several types of human tumors (Murakami & Ohigashi, 2007). The effects of PLE on the mRNA expression of COX-2 and iNOS in TNF-α-treated A549 cells were assessed using RT-qPCR. COX-2 and iNOS mRNA expression levels were determined in TNF-α-treated A549 cells incubated with various concentrations of PLE. As shown in Figure 6A, TNF-α-treated cells had significantly high COX-2 mRNA expression when compared with non-treated cells ($p < 0.001$). PLE at 100 and 200 µg/mL significantly decreased COX-2 mRNA expression in TNF-α-treated cells ($p < 0.01$). Previous reports showed that PLE, mostly containing rosmarinic acid, alleviated COX-2 expression both *in vitro* and *in vivo* (Kangwan *et al.*, 2019; Phromnoi *et al.*, 2019). These results indicate that PLE decreases TNF-α induced COX-2 mRNA expression in these cells. The inhibitory effect of PLE on COX-2 mRNA expression results in a low production of pro-inflammatory prostaglandins and reduces inflammation. As shown in Figure 6B, TNF-α slightly increased iNOS mRNA expression in A549 cells. This finding is consistent with the previous report that TNF-α did not significantly stimulate iNOS mRNA expression and NO production in A549 cells (Kwon, Newcomb, & George, 2001; Roy, Sharma, Sharma, Aggarwal, & Bose, 2004). After treating A549 cells with TNF-α and PLE, there was no difference in iNOS mRNA expression.

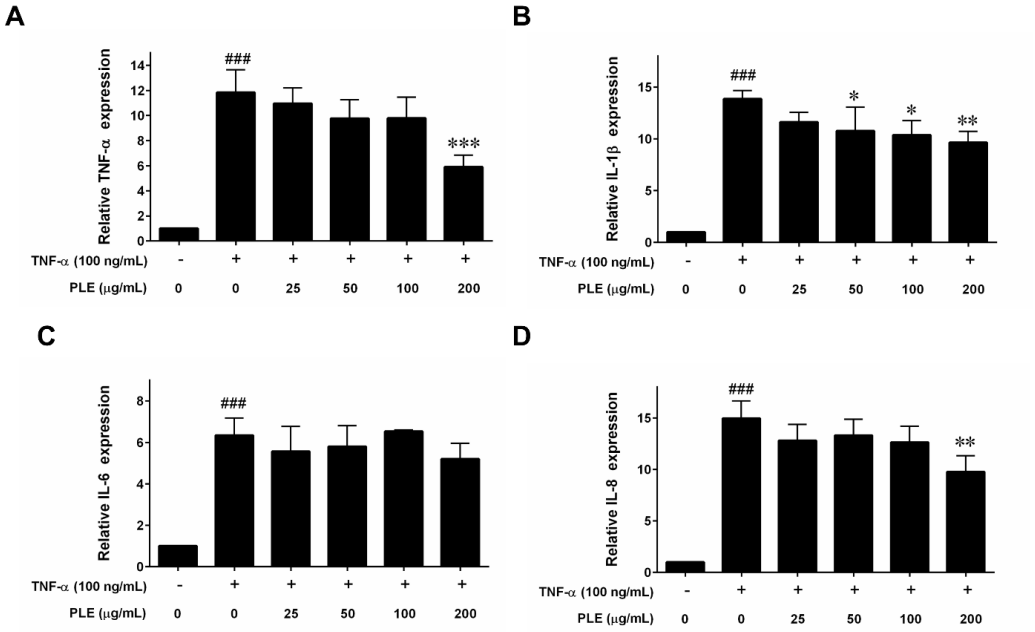

Figure 5. Effects of PLE on TNF-α (A), IL-1β (B), IL-6 (C), and IL-8 (D) mRNA expression levels were measured using RT-qPCR. TNF-α-treated A549 cells were incubated with various concentrations of PLE. All measurements were performed in triplicate. The data are presented as mean ± SD., ### $p < 0.001$ compared to non-treated cells, *$p < 0.05$ compared to TNF-α-treated A549 cells alone, **$p < 0.01$ compared to TNF-α-treated A549 cells alone, ***$p < 0.001$ compared to TNF-α-treated A549 cells alone.

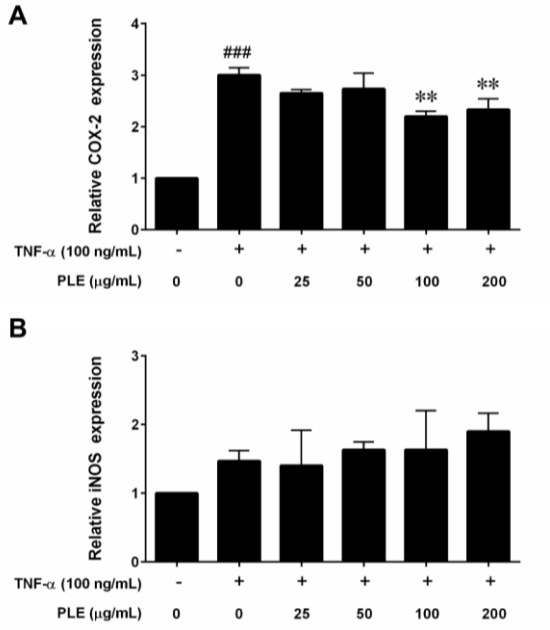

Figure 6. Effects of PLE on COX-2 (A), and iNOS (B) mRNA expression levels were measured using RT-qPCR. TNF-α-treated A549 cells were incubated with various concentrations of PLE. All measurements were performed in triplicate. The data are presented as mean ± SD., ### $p < 0.001$ compared to non-treated cells, **$p < 0.01$ compared to TNF-α-treated A549 cells alone.

4. Conclusions

Our results indicate that PLE contains polyphenols, mainly rosmarinic acid, and exhibits antioxidant and anti-inflammatory effects in TNF-α-treated A549 human lung cancer cells. PLE exhibited anti-oxidative effects against the overproduction of intracellular ROS, and this indicates an important role in preventing oxidative stress. Moreover, PLE reduced lung cancer cell inflammation by downregulating the expression of pro-inflammatory mediators, including IL-1β, IL-8, TNF-α, and COX-2. Oxidative stress and inflammation predispose to the development of various types of cancer, including lung cancer. Therefore, PLE being rich in polyphenols and rosmarinic acid exhibits antioxidant and anti-inflammatory activity, and it may be used as an alternative pharmaceutical and functional food ingredient for lung cancer prevention.

List of abbreviations

μg/mL	Microgram per milliliter
μM	Micromolar
A549	Non-small cell lung carcinoma cell line
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
$AlCl_3$	Aluminum chloride
ATCC	American Type Culture Collection
cDNA	Complementary deoxyribonucleic acid
CE	Catechin equivalent
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fe^{2+}	Ferrous ion
g	Gram
GAE	Gallic acid equivalent
h	Hour
HPLC	High performance liquid chromatography
IC_{20}	20% inhibitory concentration
IC_{50}	50% inhibitory concentration
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
MDA-MB-231	Human breast adenocarcinoma
Mg	Magnesium
mg/g	Milligram per gram
mg/mL	Milligram per milliliter
min	Minute
mL/min	Milliliter per minute
mM	Millimolar
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NF-κB	Nuclear factor-kappa B
ng⁄ml	Nanogram per milliliter
nm	Nanometer
ºC	Degree Celsius
p	p-value
PBS	Phosphate buffered saline
PLE	Perilla leaf extract
RAW 264.7	Monocyte/macrophage cell line
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Real-time reverse transcription polymerase chain reaction
SD	Standard deviation
SC_{50}	50% scavenging concentration
TFA	Trifluoroacetic acid
TFC	Total flavonoid content
TNF-α	Tumor necrosis factor-alpha
TPC	Total phenolic content

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