Artemisia lactiflora Extracts Prevent Inflammatory Responses of Human Macrophages Stimulated with Charcoal Pyrolysis Smoke

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Abstract

Artemisia lactiflora, a Chinese-origin plant, has been reported to have unique phytochemicals responsible for its medicinal properties. The growth of the agricultural industry emits air pollution, which has adverse effects on health. There are limited scientific reports on the biological activities of *A. lactiflora*. Studies on its activities and mechanisms may provide insight into its use in medicinal purposes to treat those health problems and conditions. In this study, leaves of *A. lactiflora* were extracted and fractioned with solvents of different polarities. Total phenolics, total flavonoids DPPH$^•$ scavenging, ABTS$^{•+}$ scavenging, and cytotoxicity of *A. lactiflora* were assessed. Anti-inflammatory activities were evaluated by pre-treating macrophages with extract or fractions then induced inflammatory response by coconut shell pyrolysis smoke. Inflammatory responses were assessed by measuring pro-inflammatory genes expression and pro-inflammatory cytokines secretion. Among all extract and fractions of *A. lactiflora*, butanol fraction has the highest phenolic, flavonoid, and DPPH$^•$ scavenging activity. All extract and fractions significantly down-regulated pro-inflammatory genes expression (*RelA, TNF, IL6*) and decreased pro-inflammatory cytokines secretion (TNF-α, IL-6), $p <$ 0.0001, compared with pyrolysis smoke-induced macrophages. The ethyl acetate fraction showed the highest anti-inflammatory activity in decreasing pro-inflammatory cytokines secretion. These results may prove the anti-inflammatory activities of *A. lactiflora* through the inhibition of the NF-κB-dependent pathway. Taken together, this study first reported the anti-inflammatory activities of *A. lactiflora*. Thus, the plant can be used to prevent and treat inflammatory responses caused by highly oxidative pyrolysis smoke released from the re-utilization of agro-industrial leftovers.

Keywords

artemisia lactiflora, anti-inflammatory, pyrolysis smoke

Introduction

Medicinal plants have been used as new trends for complementary and alternative medicine. Phytochemicals found in plants have shown various biological activities including antioxidant, anti-inflammation, anti-diabetic, and anti-cancer properties. *Artemisia lactiflora,* a Chinese-origin medicinal plant, has been reported to have unique phytochemicals including balanophonin, aurantiamide, and isovitexin.[1] Its medicinal relatives *A. annua*, *A. argyi*, and *A. vulgaris*, which have anti-malarial,[2] anti-gastric ulcer,[3] and anti- gynecological diseases.[4] *A. lactiflora*

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may have decent medicinal activities and might be benefit for patient with those undesirable diseases and conditions.

Production of coconut shell charcoal, as the re-utilization of agro-industrial leftovers, releases toxic gasses such as carbon monoxide, nitric oxide, and aldehydes, which become air pollution. The pollution is the main problem found mostly in the agricultural area, especially in developing countries, where effective pollution management is not available. Inhalation of those toxic gasses may damage cells, induce inflammatory responses, increase respiratory tract infections,[5,6] promote the development of serious diseases, and cause long-term health problems.[6]

There are limited previous reports associated with *A. lactiflora* activities. Most of the available data consists of non-scientific, unclear information. The aims of this study were to fractionate *A. lactiflora* leaves for testing of phytochemical composition and antioxidant activities. Then we investigated anti-inflammatory activities of *A. lactiflora* extract and fractions on human coconut shell pyrolysis smoke-stimulated human macrophages. These findings may provide insight into its safety use as a food ingredient or precisely use in medicinal purposes.

Methods

Preparation of Plant Materials

Fresh leaves of *Artemisia lactiflora* Wall. ex DC. (White Mugwort) were collected from cultivation garden in Phop Phra, Tak, Thailand. Voucher samples were deposited at Botanical Garden, Walailak University, Nakhon Si Thammarat, Thailand, assigned herbarium numbers: 01545–01548.

Extraction and Fractionation of A. lactiflora Leaves

A. lactiflora leaves were washed with deionized water, absorbed with paper towels, weighed, and extracted by homogenization-maceration. The leaves of 1.4 kg were homogenized and macerated with seven liters of 1% acetic acid in 50% methanol at 4 °C, for 7 days with agitation. The extract was filtered through 0.22 μM polyethersulfone membrane and concentrated at 40 °C. The extract was dissolved with 500 mL of deionized water and fractionated by liquid-liquid partitioning using solvents of different polarity (RCI Labscan Ltd, Bangkok, Thailand). The aqueous crude extract was first fractionated three times with equal volume of diethyl ether to obtain non-polar molecules. Sodium bicarbonate was added to the partitioned aqueous extract to reach pH of 8.5, to convert phenolic acid to soluble phenolic sodium salts. The alkaline aqueous extract was then fractionated three times with chloroform to separate non-phenolic molecules. The pH of the aqueous was adjusted to 3.5, to convert phenolic sodium salts back to phenolic acid. The acidic aqueous extract was partitioned three times with ethyl acetate to fractionate phenolic acid molecules. Residual phenolics were further fractionated three times with n-butanol, separated from aqueous residual fraction. Extract and fractions of *A. lactiflora* leaves were dried by evaporation, weighted, stored in airtight tubes, and stored in a desiccator.[7,8]

Determination of Total Phenolic and Flavonoid Contents

Total phenolic content was determined by Folin-Ciocalteu method using 96-well plate. Reaction contains 100 μL of 1 mg/mL test substance, 100 μL of 10% Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany), and 100 μL of 0.1 M sodium carbonate. The reaction was mixed, incubated in the dark at room temperature for 1 h, and measured for the absorbance at 750 nm using microplate reader (Eon Microplate Spectrophotometer, BioTek Instruments, Winooski, VT, USA). Total phenolic of test substances was calculated from the standard curve of gallic acid as mg gallic acid equivalent/g of dry extract.[9,10]

Total flavonoid content was determined by aluminum chloride colorimetric method using 96-well plate. The reaction mixed 100 μL of 1 mg/mL test substance with 100 μL of 2% aluminum chloride in methanol (Merck KGaA, Darmstadt, Germany), incubated in the dark at room temperature for 30 min, and measured for the absorbance at 415 nm using microplate reader. Total flavonoid of test substances was calculated from the standard curve of quercetin as mg quercetin equivalent/g of dry extract.[9,10]

Determination of Antioxidant Activities

Antioxidant activities of *A. lactiflora* extract and fraction were tested against free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) and anion radical of 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) (Merck KGaA, Darmstadt, Germany).[9,10]

For DPPH assay, 20 μL of each 1 mg/mL test substance was mixed with 180 μL of 0.1 mM DPPH$^\bullet$ in methanol, incubated in the dark at room temperature for 30 min, and measured at the absorbance of 517 nm using microplate reader.

For ABTS assay, the cation was generated by oxidizing 7 mM ABTS with 2.45 mM potassium persulphate at the ratio of 2:3, incubated in the dark for 16 h, and diluted with methanol to the absorbance of 0.7 at 734 nm. The activity of test substance was tested by mixing 20 μL of each 1 mg/mL test substance and 180 μL of prepared ABTS$^{\bullet+}$ solution, incubated in the dark at room temperature for 45 min, and measured at the absorbance of 734 nm using microplate reader.

Percent scavenging activities of each extract against DPPH$^\bullet$ and ABTS$^{\bullet+}$ were calculated in comparison with the positive control, ascorbic acid ($C_6H_8O_6$), using the equation: Percent scavenging activity = (absorbance of standard - subtracted absorbance of sample) ÷ absorbance of standard × 100.[9,10]

Collection of Coconut Shell Charcoal Pyrolysis Smoke

Coconut shell pyrolysis smoke deposited on exhaust tubes of the charcoal kiln was collected from the production plant in Tha Sala, Nakhon Si Thammarat, Thailand. Pyrolysis smoke aggregates were kept in an airtight container until experimentation.

Ethical Approval, Biosafety, and Blood Sample Collection

Methods and protocols involving human samples were approved by the Human Research Ethics Committee of Walailak University, project number: WU-EC-AH-2-129-63. Processing of biological specimens was approved by the Biosafety Committee of Walailak University, clearance number: WU-IBC-63-006. Leukocyte-rich blood component (buffy coat) was obtained from Maharaj Nakhon Si Thammarat Hospital, Thailand with permission.

Isolation and Differentiation of Human Peripheral Blood Monocytes

Human monocytes were isolated from buffy coat (leukocyte concentrate) by density gradient centrifugation followed by size sedimentation centrifugation.[7] Hank's balanced salt solution (HBSS)-diluted buffy coat of 10 mL was layered on 5 mL of Lymphoprep solution (Alere Technologies AS, Oslo, Norway) in 15 mL centrifuge tube and centrifuged at 3000 rpm for 30 min. Separated layer of peripheral blood mononuclear cells was collected and suspended with HBSS. Cells suspension of 5 mL were then layered on 10 mL of 46% Percoll solution (Ge Healthcare Bio-Sciences AB, Uppsala, Sweden) in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 3000 rpm for 30 min. Isolated cells were washed with HBSS and suspended in completed RPMI medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, USA). Monocytes were differentiated into macrophages by plating onto cell culture flask and incubated at 37 °C in humidified condition with 5% carbon dioxide for 7 days.

Determination of Cellular Cytotoxicity

Cytotoxicity of *A. lactiflora* extract, fractions, and pyrolysis smoke was determined by neutral red uptake cytotoxicity bioassay.[7,11] Each test substance was dissolved in dimethyl sulfoxide (DMSO) and diluted with completed-RPMI medium to 0–10000 µg/mL geometrically sequential concentrations. Human macrophages were plated onto a 96-well plate at the density of 5×10^4 cells/well, rested for 48 h, and washed once with HBSS. Cells were then treated with 50 µL of the sequentially diluted test substance for 24 h, washed with HBSS, and stained with 50 µL of 50 µg/mL neutral red dye in RPMI for 2 h. Cells were washed and dissolved with 50 µL of acid-alcohol solution. Absorbance at 545 nm of each condition was measured using microplate reader.

Percentage of cell viability of test substance-treated cells was calculated by comparing with untreated control condition. Data were plotted and analyzed by dose-response curve fitting to calculate median lethal concentration (LC_{50}) and 5% lethal concentration (LC_5) of the test substance.

Investigation of Anti-Inflammatory Activities

Human macrophages were plated onto 24-well plate at the density of 5×10^5 cells/well, rested for 48 h, and washed once with HBSS. Test substances were dissolved in DMSO and diluted with completed RPMI medium. Cells were pre-treated with 500 µL of LC_5 concentration of *A. lactiflora* extract or fractions for 6 hours, washed once with HBSS, and stimulated with 500 µL of LC_5 concentration of pyrolysis smoke. Lipopolysaccharide (LPS) (Merck KGaA, Darmstadt, Germany) at a concentration of 10 ng/mL served as a positive control in stimulating pro-inflammatory response. Non-steroidal anti-inflammatory drug, 5.42 µg/mL of aspirin (SEA Pharm, Co., Ltd, Phra Nakhon Si Ayutthaya, Thailand), and steroidal anti-inflammatory drug, 12.5 ng/mL of prednisolone (Medic Pharma Co., Ltd, Samut Sakhon, Thailand), served as control drugs for anti-inflammatory activities.[12]

Cell culture supernatant was collected to microcentrifuge tube for the measurement of secreted pro-inflammatory cytokines by ELISA. Treated cells were processed to RNA extraction for the quantification of pro-inflammatory genes expression by qRT-PCR.

RNA Extraction and Quantitative Reverse Transcription PCR (qRT-PCR)

For the total RNA extraction, cells in each well of 24-well plate were washed once with HBSS, lysed with 200 µL of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), transferred to 1.5 mL microcentrifuge tube, and incubated at room temperature for 5 min. Reagent phases were separated by adding 50 µL of chloroform to the tube, mixed by inverting, incubated for 3 min, and centrifuged for 15 min at $12000 \times g$, 4 °C. Aqueous phase containing RNA was transferred to new tube, then RNA was precipitated by adding 100 µL of isopropanol, mixed for 10 min, and centrifuged for 10 min at $12000 \times g$, 4 °C. Supernatant was discarded, RNA pellet was washed with 200 µL of 75% ethanol, mixed by vortexing, and centrifuged for 5 min at $7500 \times g$, 4 °C. Supernatant was discarded, ethanol was allow to evaporate, and RNA was dissolved with RNase-free water (Merck KGaA, Darmstadt, Germany). RNA concentration was directly quantified using NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).[7]

For the quantification of mRNA expression, each reaction contains 4 µL of 1 ng/mL RNA template, 0.8 µL of 10 µM forward primer, 0.8 µL of 10 µM reverse primer 0.2 µL of reverse transcriptase, 0.4 µL of RNase inhibitor, and 10 µL of SensiFAST SYBR No-ROX One-Step reagent (Bioline Reagents Ltd, London, UK). One-step qRT-PCR steps were reverse transcription at 45 °C for 10 min, polymerase activation at 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 5 sec with annealing/extension at 60 °C for 20 sec. Forward and reverse primer for each pro-inflammatory gene was retrieved from PrimerBank database (Table 1).[13] Expression of each gene was compared with expression of beta-actin gene (*ACTB*) and calculated by normalized gene expression analysis using $2^{-\Delta\Delta Ct}$ method.[14]

Enzyme-Linked Immunosorbent Assay (ELISA)

Level of pro-inflammatory cytokines, TNF-α and IL-6, secreted from human macrophages was quantified by ELISA using antibody pairs (ImmunoTools, Friesoythe, Germany) and reagent kit (GenPack-5, MabTag GmbH, Friesoythe, Germany). ELISA plate was coated with 100 µL of capture antibody for 24 h at room temperature and blocked with 300 µL of blocking buffer for 1 h. Cell culture supernatant of 100 µL was added to ELISA well along with cytokine standard and incubated at room temperature for 2 h with shaking. Plate was washed 5 times with phosphate-buffered saline containing 0.05% tween-20 (PBST), 100 µL of biotinylated detection antibody was added and incubated at room temperature for 2 h with shaking. The plate was washed, 100 µL of streptavidin-HRP was added and incubated in the dark at room temperature for 30 min with shaking. Plate was washed, 100 µL of TMB substrate was added, and incubated in the dark at room temperature for 30 min with shaking. The reaction was stopped by adding 50 µL of stop solution. Absorbance at 450 nm of the reaction was measured by microplate reader. Cytokine level of each experimental condition was calculated according to the standard curve of the reference standard cytokine.

Statistical Analysis and Study Report

This study was performed in a triplicate of three-independent experiments. Data was collected, analyzed, and plotted by GraphPad Prism

Table 1. Oligonucleotide Primers Used in This Study.

Gene	Primer	Oligonucleotide Sequence, 5′ to 3′
RelA	Forward	ATGTGGAGATCATTGAGCAGC
Homo sapiens v-rel reticuloendotheliosis viral oncogene homolog A (avian), mRNA	Reverse	CCTGGTCCTGTGTAGCCATT
TNF	Forward	CCTCTCTCTAATCAGCCCTCTG
Homo sapiens tumor necrosis factor, mRNA	Reverse	GAGGACCTGGGAGTAGATGAG
IL6	Forward	ACTCACCTCTTCAGAACGAATTG
Homo sapiens interleukin 6 (interferon, β2), mRNA	Reverse	CCATCTTTGGAAGGTTCAGGTTG
ACTB	Forward	CATGTACGTTGCTATCCAGGC
Homo sapiens β actin; β cytoskeletal actin, mRNA	Reverse	CTCCTTAATGTCACGCACGAT

(GraphPad Software, San Diego, CA, USA). Distribution of data was analyzed by Shapiro-Wilk test. Differences among data sets was analyzed by one-way ANOVA with Bonferroni multiple comparisons (normal distribution) or Kruskal-Wallis test with Dunn's multiple comparisons (non-parametric) at a significance level of 0.05. Detailed statistical analyses are available in the Supplementary File.

Experimental data was reported according to the guideline for Reporting Experiments in Homeopathic Basic Research (REHBaR) as suggested by EQUATOR Library of Reporting Guidelines.[15]

Results

A. lactiflora Leaves Extract and Fractions

Extraction of 1400 g fresh *A. lactiflora* leaves yielded 55.63 g (3.97%) of crude extract. Fractionation of the crude extract yielded 1.58 g (0.11%) of diethyl ether fraction, 1.08 g (0.08%) of chloroform fraction, 1.03 g (0.07%) of ethyl acetate fraction, 1.29 g (0.09%) butanol fraction, and 46.93 g (3.35%) of residual salt fraction.

Total Phenolic and Total Flavonoid Contents of A. lactiflora Leaves Extract and Fractions

Total phenolic content of *A. lactiflora* extract and fractions are shown in Figure 1A. Crude extract had total phenolics of 8.88 ± 0.72 mg gallic acid equivalent/g of dry extract. Butanol fraction had the highest total phenolics of 14.77 ± 0.82 mg gallic acid equivalent/g of dry extract, significantly higher than crude extract ($p < 0.0001$). Ethyl acetate fraction also had higher phenolic contents than crude extract at 13.72 ± 0.99 mg gallic acid equivalent/g of dry extract ($p < 0.0001$). There was no significant difference between total phenolics of butanol and ethyl acetate fractions. Lower phenolic content was shown in diethyl ether, chloroform, and residual fraction at 2.49 ± 0.21, 2.98 ± 0.25, and 2.90 ± 0.22 mg gallic acid equivalent/g of dry extract, respectively, compared with crude extract ($p < 0.0001$). There was no significant difference among total phenolics of diethyl ether, chloroform, and residual fractions.

Total flavonoid content of *A. lactiflora* extract and fractions are shown in Figure 1B. Crude extract had the lowest total flavonoid of 0.53 ± 0.02 mg quercetin equivalent/g of dry extract. Butanol fraction had the highest total flavonoid of 2.27 ± 0.20 mg quercetin equivalent/g of dry extract, significantly higher than crude extract ($p < 0.0001$). Significantly higher total flavonoid content than crude extract was shown in diethyl ether ($p < 0.0001$), residual ($p < 0.0001$), chloroform ($p < 0.0001$), and ethyl acetate fractions ($p = 0.0007$) at 1.92 ± 0.17, 1.4 ± 0.14, 1.22 ± 0.09, and 0.97 ± 0.10 mg quercetin equivalent/g of dry extract, respectively. There was no significant difference in total flavonoid content when comparing chloroform with ethyl acetate fractions and chloroform with residual fractions.

Antioxidant Activity of A. lactiflora Leaves Extract and Fractions

Percentage of DPPH• free radical scavenging activity of *A. lactiflora* extract and fractions are shown in Figure 2A. Ascorbic acid had DPPH• free radical scavenging activity of $96.37 \pm 0.72\%$. Crude extract had significantly lower DPPH• free radical scavenging activity than the ascorbic acid at $78.76 \pm 6.68\%$ ($p < 0.0001$). Significantly higher activity was observed in butanol fraction at $84.15 \pm 1.48\%$, $p = 0.0053$, compared with crude extract. Significantly lower activity than crude extract was observed in ethyl acetate ($p = 0.0006$), residual ($p < 0.0001$), diethyl ether ($p < 0.0001$), and chloroform fractions ($p < 0.0001$) at $72.28 \pm 3.52\%$, $25.77 \pm 1.95\%$, $11.80 \pm 1.03\%$, and $6.84 \pm 0.64\%$, respectively. There were significant differences of DPPH• free radical scavenging activity among all extract and fractions ($p < 0.05$).

Percentage of ABTS•+ radical cation scavenging activity of *A. lactiflora* extract and fractions are shown in Figure 2B. Ascorbic acid had ABTS•+ free radical scavenging activity of $93.48 \pm 0.15\%$. The highest ABTS•+ radical cation scavenging activity was found in crude extract at $96.02 \pm 0.83\%$, significantly higher than ascorbic acid ($p = 0.0106$). Fractions of the extract had significantly lower activity than the crude extract ($p \leq 0.0001$). Butanol, ethyl acetate, chloroform, diethyl ether, and residual fractions had the scavenging activity of $92.91 \pm 0.32\%$, $92.71 \pm 0.25\%$, $81.88 \pm 1.19\%$, $70.84 \pm 2.82\%$, and $70.36 \pm 1.00\%$, respectively. There was no significant difference of ABTS•+ radical cation scavenging activity when comparing diethyl ether with residual fractions and ethyl acetate with butanol fractions.

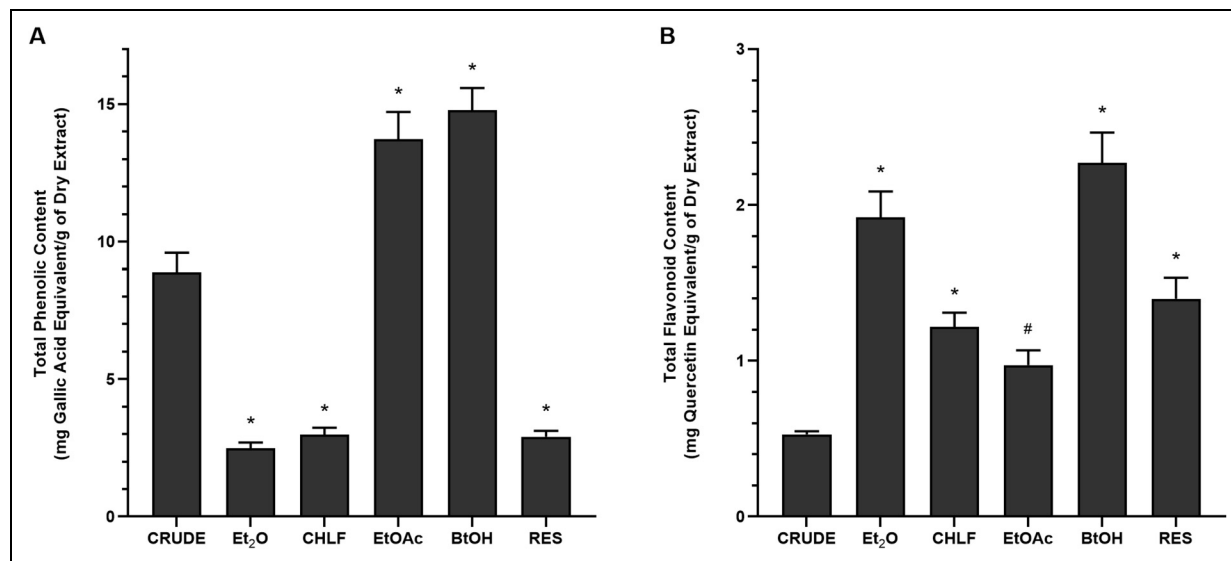**Figure 1.** Total phenolic and total flavonoid contents of *A. lactiflora* leaves extract and fractions. (A) Total phenolic content. (B) Total flavonoid content. Abbreviation: CRUDE, crude extract; Et$_2$O, diethyl ether fraction; CHLF, chloroform fraction; EtOAc, ethyl acetate fraction; BtOH, butanol fraction; RES, residual salt fraction. One-way ANOVA, compared with CRUDE: *, $p < 0.0001$; #, $p = 0.0007$.

Cellular Cytotoxicity of *A. lactiflora* Leaves Extract, Fractions, and Pyrolysis Smoke

Median lethal concentration (LC$_{50}$) of test substances is shown in Figure 3. Butanol fraction of *A. lactiflora* leaves extract had the highest LC$_{50}$ of 3899.42 μg/mL while diethyl ether fraction had the lowest LC$_{50}$ of 18.58 μg/mL. Crude extract, residual, ethyl acetate, and chloroform fractions had LC$_{50}$ of 2747.89, 2133.04, 1415.79, and 96.16 μg/mL, respectively. Coconut shell charcoal pyrolysis smoke had a moderate LC$_{50}$ of 2546.83 μg/mL.

Five percent lethal concentration (LC$_5$) of test substances is also shown in Figure 3. *A. lactiflora* crude extract, diethyl ether, chloroform, ethyl acetate, butanol, aqueous residual fractions, and pyrolysis smoke had LC$_5$ of 1209.77, 7.24, 25.8, 356.03, 51.67, 1189.68, and 1118.17 μg/mL, respectively. These LC$_5$ was used in the investigation of the inflammatory response of pyrolysis smoke and anti-inflammatory activities of *A. lactiflora* extract and fractions.

Inflammatory Potential of Charcoal Pyrolysis Smoke and Anti-Inflammatory Activities of *A. lactiflora* Extract

Inflammatory responses of macrophages were investigated by measuring pro-inflammatory genes expression (Figure 4) and pro-inflammatory cytokines secretion (Figure 5).

The potent inflammatory stimulator, LPS, significantly up-regulated expression of *RelA*, *TNF*, and *IL6* to 3.72 ± 0.15, 4.29 ± 0.66, and 4.82 ± 0.47 -fold of untreated control macrophages ($p < 0.0001$) (Figure 4). Coconut shell pyrolysis smoke had comparable activity as LPS in significantly up-regulated expression of *RelA*, *TNF*, and *IL6* to 2.91 ± 0.23, 3.81 ± 0.69, and 4.15 ± 0.35 -fold of untreated control

macrophage ($p < 0.0001$). There was only significantly lower *RelA* expression ($p < 0.0001$) in cells stimulated between LPS and pyrolysis smoke. *A. lactiflora* extract, diethyl ether, chloroform, ethyl acetate, butanol, and residual fractions significantly down-regulated expression of *RelA* to 1.41 ± 0.07, 1.28 ± 0.10, 1.49 ± 0.11, 1.32 ± 0.20, 1.46 ± 0.23, and 1.49 ± 0.11 -fold, respectively, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells (Figure 4A). The extract and fractions significantly down-regulated *TNF* to 1.57 ± 0.18, 1.39 ± 0.15, 1.89 ± 0.22, 1.60 ± 0.19, 1.80 ± 0.15, and 1.42 ± 0.11 -fold, respectively, $p < 0.0001$ (Figure 4B). The extract and fractions significantly down-regulated *IL6* to 1.92 ± 0.22, 1.67 ± 0.22, 2.54 ± 0.43, 1.87 ± 0.29, 2.12 ± 0.27, and 2.26 ± 0.26 -fold, respectively, $p < 0.0001$ (Figure 4C). There was no significant difference in activity among extract and fractions of *A. lactiflora*. The lowest down-regulation of *RelA*, *TNF*, and *IL6* was observed in cells pre-treated with diethyl ether fraction, compared with pyrolysis smoke-stimulated cells. The activity of *A. lactiflora* extract and fractions was comparable to the activity of inflammatory drugs, aspirin and prednisolone, in down-regulation of pro-inflammatory genes. Aspirin significantly down-regulated expression of *RelA*, *TNF*, and *IL6* to 1.18 ± 0.14, 1.67 ± 0.20, and 1.94 ± 0.30 -fold, respectively, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells. Prednisolone significantly down-regulated expression of *RelA*, *TNF*, and *IL6* to 1.14 ± 0.12, 1.45 ± 0.15, and 1.70 ± 0.19 -fold, respectively, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells.

Baseline secretion of TNF-α and IL-6 from untreated control macrophages was 264.80 ± 13.60 and 345.10 ± 8.17 ng/mL (Figure 5). LPS significantly increased secretions of TNF-α and IL-6 to 1127.00 ± 10.70 and 1427.00 ± 8.60 ng/mL, $p < 0.0001$, compared with untreated control macrophages. Pyrolysis smoke significantly increased secretion of TNF-α
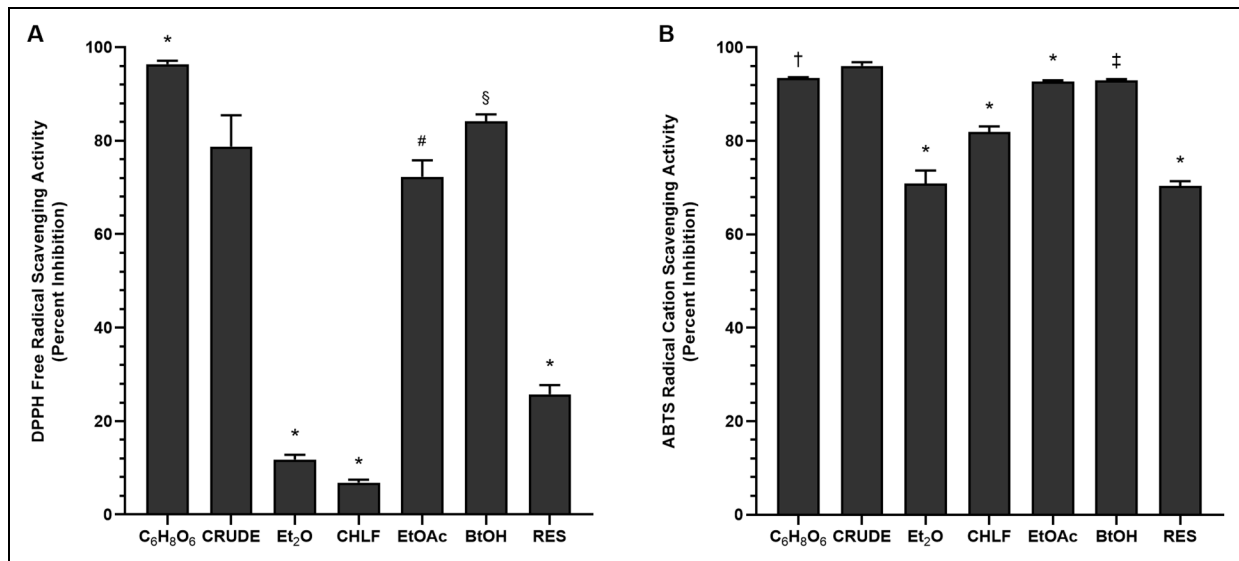
Figure 2. Antioxidant activities of *A. lactiflora* leaves extract and fractions. (A) DPPH˙ free radical scavenging activity. (B) ABTS˙⁺ radical cation scavenging activity. Abbreviation: $C_6H_8O_6$, ascorbic acid; CRUDE, crude extract; Et_2O, diethyl ether fraction; CHLF, chloroform fraction; EtOAc, ethyl acetate fraction; BtOH, butanol fraction; RES, residual salt fraction. One-way ANOVA, compared with CRUDE: *, $p < 0.0001$; #, $p = 0.0007$; §, $p = 0.0088$; †, $p = 0.0106$; ‡, $p = 0.0001$.

and IL-6 to 866.20 ± 22.41 and 1102.00 ± 9.64 ng/mL, $p < 0.0001$, compared with untreated control macrophages. The inflammatory activity of pyrolysis smoke was significantly lower than the activity of LPS ($p < 0.0001$). *A. lactiflora* extract, diethyl ether, chloroform, ethyl acetate, butanol, and residual fractions significantly decreased secretion of TNF-α to 730.90 ± 18.05, 501.20 ± 18.54, 365.90 ± 21.40, $71.55 \pm$

2.68, 708.60 ± 15.71, and 696.60 ± 15.42 ng/mL, respectively, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells (Figure 5A). The extract and fractions significantly decreased secretion of IL-6 to 578.20 ± 14.15, 444.40 ± 11.38, 381.20 ± 13.13, 173.80 ± 4.08, 892.60 ± 15.84, and 942.00 ± 19.55, respectively, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells (Figure 5B). There were significant differences
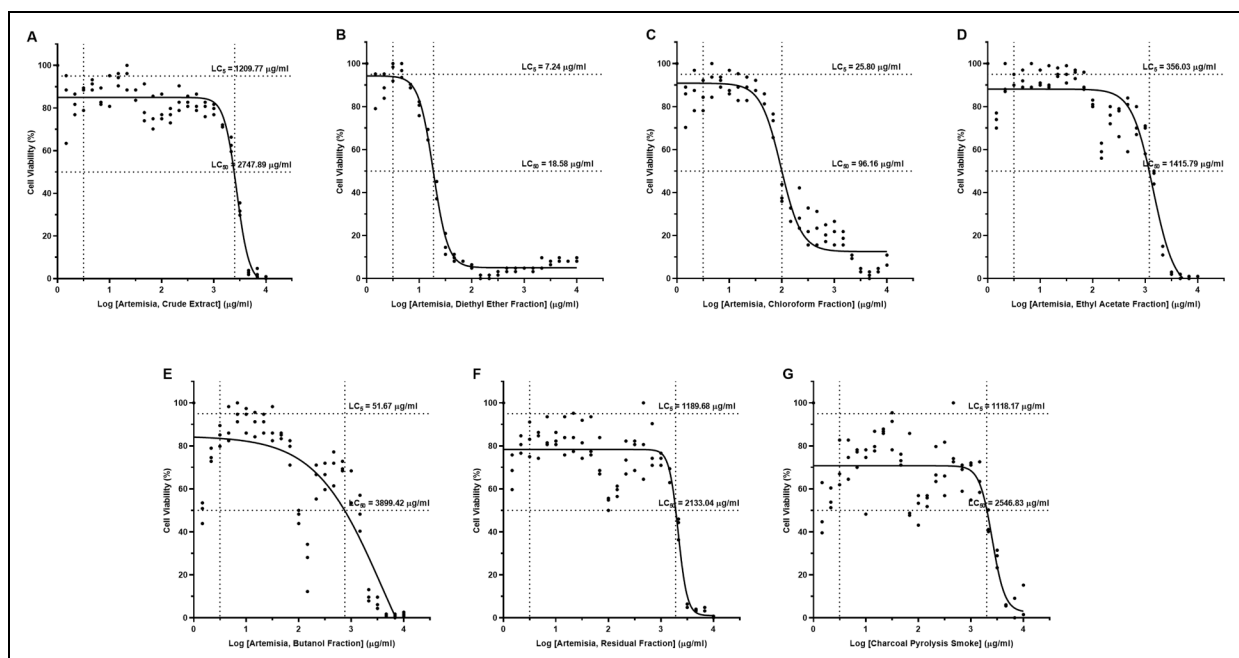

Figure 3. Dose-response curve showing LC_5 of test substances. (A) Crude extract of *A. lactiflora* leaves. (B) diethyl ether fraction. (C) chloroform fraction. (D) ethyl acetate fraction. (F) butanol fraction. (G) coconut shell pyrolysis smoke.
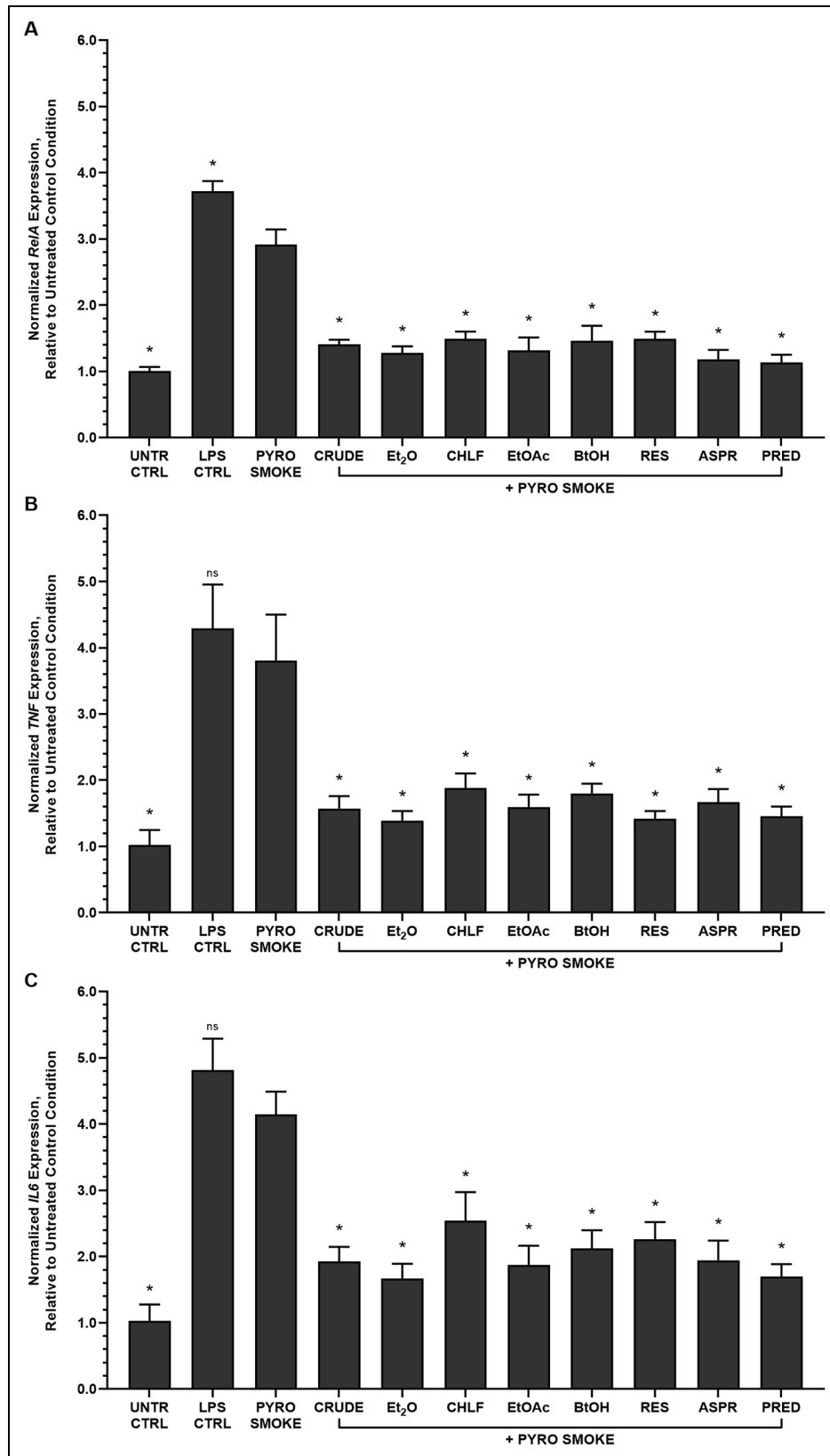
Figure 4. Expression of pro-inflammatory genes (A) *RelA*, (B) *TNF*, and (C) *IL6*. Cells were stimulated with charcoal pyrolysis smoke to induce pro-inflammatory response. *A. lactiflora* extract and fractions were used to pre-treat cells to investigate anti-inflammatory activities. Abbreviation: UNTR CTRL, untreated control; LPS, lipopolysaccharide; PYRO, pyrolysis smoke; CRUDE, crude extract; Et_2O, diethyl ether fraction; CHLF, chloroform fraction; EtOAc, ethyl acetate fraction; BtOH, butanol fraction; RES, residual salt fraction; ASPR, aspirin; PRED, prednisolone. One-way ANOVA, compared with PYRO SMOKE-stimulated cells: *, $p < 0.0001$; ns, non-significant.
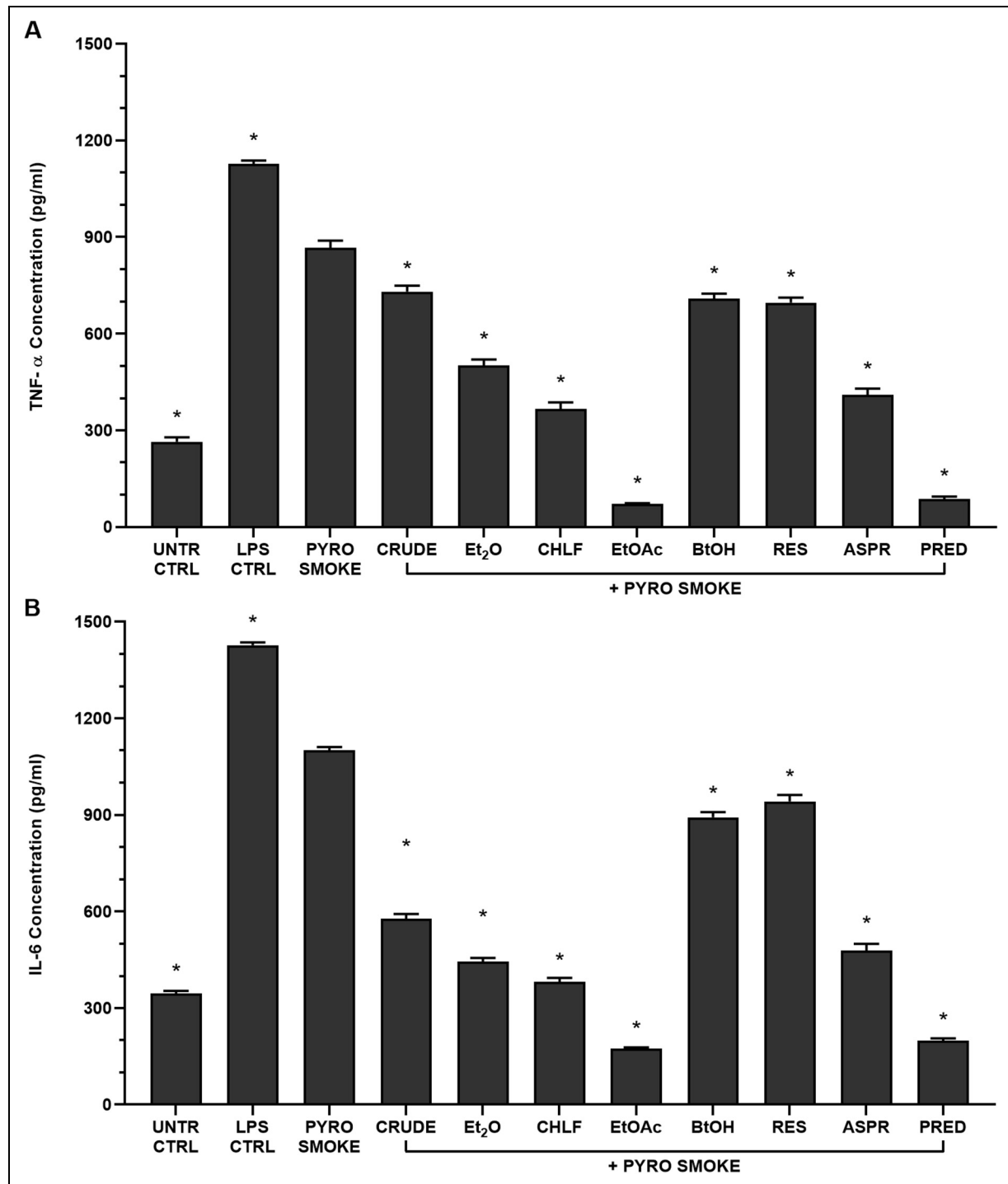
Figure 5. Secretion of pro-inflammatory cytokines (A) TNF-α, and (B) IL-6. Cells were stimulated with charcoal pyrolysis smoke to induce pro-inflammatory response. *A. lactiflora* extract and fractions were used to pre-treat cells to investigate anti-inflammatory activities. Abbreviation: UNTR CTRL, untreated control; LPS, lipopolysaccharide; PYRO, pyrolysis smoke; CRUDE, crude extract; Et$_2$O, diethyl ether fraction; CHLF, chloroform fraction; EtOAc, ethyl acetate fraction; BtOH, butanol fraction; RES, residual salt fraction; ASPR, aspirin; PRED, prednisolone. One-way ANOVA, compared with PYRO SMOKE-stimulated cells: *, $p < 0.0001$.

in secretion of TNF-α and IL-6 among cells pre-treated with most of extract and fractions of *A. lactiflora* ($p \leq 0.0003$). No significant difference in secretion of TNF-α between cells pre-treated with crude extract and butanol fraction as well as

between crude extract and residual fraction. The lowest TNF-α and IL-6 secretion was observed in cells pre-treated with ethyl acetate fraction, compared with pyrolysis smoke-stimulated cells. The activity of *A. lactiflora* extract and

fractions was comparable to the activity of inflammatory drugs, aspirin and prednisolone in decreasing secretion of pro-inflammatory cytokines. Aspirin significantly decreased secretion of TNF-α and IL-6 to 410.40 ± 19.46 and 479.40 ± 20.07, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells. Prednisolone significantly decreased secretion of TNF-α and IL-6 to 86.55 ± 7.85 and 199.00 ± 7.30, $p < 0.0001$, compared with pyrolysis smoke-stimulated cells.

Discussion

Medicinal plants in the genus *Artemisia* proved to have various health benefits. The well-known *A. annua* comprises of the anti-malarial biomolecule, artemisinin.[2] The traditional Chinese *A. argyi* is notable for using as a remedy for gastric ulcers.[3] The common *A. vulgaris* has been used to treat immunological and gynecological diseases.[4] However, few studies have reported only phytochemical composition and anti-cancer of *A. lactiflora*.[1,16,17] Thus, this study tends to investigate antioxidant and anti-inflammatory activities of *A. lactiflora* on innate immune cells exposed to inflammatory substances.

Extraction and fractionation of *A. lactiflora* yielded extracts with different properties as described by the fractionation method.[7,8] The liquid-liquid partitioning method is well-practiced in many studies for fractionating different polarities of phytochemicals by solvents.[8,18,19] We obtained crude extract (concentrate), diethyl ether (non-polar), chloroform (non-phenolic), ethyl acetate (low-polar polyphenolic), butanol (high polar polyphenolic), and aqueous residual (salts) fractions. Butanol fraction showed the highest total phenolics, total flavonoids, and DPPH• free radical scavenging activity (Figure 1–2). Other fractions showed different levels of phenolics, flavonoids, DPPH•, and ABTS•+ scavenging activities due to their composition obtained from the fractionation method. Extraction of Artemisia with aqueous-methanol can obtain phytochemicals with the highest antioxidant activities among other aqueous-alcohol-based solvent systems, as demonstrated in a previous study.[20] Our *A. lactiflora* crude extract had lower total phenolic and total flavonoid contents while showing higher radical scavenging activity compared with the well-known *A. annua* extract of the previous studies.[21,22]

Cytotoxicity of test substances can be evaluated by various in vitro bioassays. This study used the neutral red uptake assay, which is more sensitive than tetrazolium salt or enzymatic-based assays.[11] Viability of treated cells was calculated for LC_{50} (IC_{50}) by dose-response curve fitting. Among extract and fractions of *A. lactiflora*, diethyl ether fraction, with the lowest polarity of compounds, had the highest cytotoxicity. In contrast, butanol fraction, with the highest polarity of compounds, had the lowest cytotoxicity (Figure 3). These results may indicate the property of different polar compounds in having different activities and cytotoxicity since the solvents in all extract and fractions were completely evaporated.[23,24] When comparing IC_{50} among *Artemisia* extracts, our *A. lactiflora* crude extract, tested on primary human macrophages, had lower toxicity than crude extracts of *A. annua*,[25] *A. absinthium*,[26] *A. vulgaris*,[26] and *A. biennis*[27] tested in leukemic or cancer cell lines. Pyrolysis smoke of plant materials contains mainly tar and other carbon-based oxidants.[28] Our coconut shell pyrolysis smoke had moderate cytotoxicity comparable to the crude extract of *A. lactiflora* (Figure 3G).

Prevention and treatment of respiratory tract diseases caused by exposure to air pollution, cigarette smoking, and viral infection are currently interested.[29,30] In vitro model mimicking the inflammatory response of alveolar macrophages, the crucial immune cells in response to inhalable toxic substances and viral particles, was selected in this study. We isolated macrophages from blood of healthy donors and differentiated them into macrophages. The macrophages were then treated with highly oxidative charcoal pyrolysis smoke, the common air pollution of agro-industry with toxic gasses and chemicals,[5,6] to simulate those inflammatory response in the lung. The pyrolysis smoke has been found to significantly induced inflammatory response of macrophages by upregulating expression of pro-inflammatory genes including *RelA* (NF-κB p65), *TNF*, and *IL6* ($p < 0.0001$, compared with untreated control). It correspondingly increased the secretion of pro-inflammatory cytokines: TNF-α and IL-6 ($p < 0.0001$, compared with untreated control) (Figure 4–5). The inflammatory activities of the pyrolysis smoke were comparable with the standard oxidative stress, inflammatory inducer, LPS. These results showed that the pyrolysis smoke-induced inflammatory response via the activation of the NF-κB-dependent inflammatory signaling pathway.[31]

Pre-treating of macrophages with *A. lactiflora* extract and fractions prior to inducing inflammatory response by pyrolysis smoke demonstrated anti-inflammatory activities. All extract and fractions significantly down-regulated expression of pro-inflammatory genes (*RelA*, *TNF*, *IL6*) and decreased secretion of pro-inflammatory cytokines (TNF-α, IL-6), $p < 0.0001$, compared with pyrolysis smoke-induced macrophage (Figure 4–5). Anti-inflammatory activities of *A. lactiflora* extract and fractions were correlated with activities of anti-inflammatory drugs, aspirin and prednisolone. There was no difference in activities of extract and fractions in expression of pro-inflammatory genes while variations in secretion of pro-inflammatory cytokines was observed. The ethyl acetate fraction of *A. lactiflora* extract exhibited the most significant pro-inflammatory cytokines secretion as strong as the steroidal anti-inflammatory drug, prednisolone (Figure 4–5). These results indicated that *A. lactiflora* extract and fractions had anti-inflammatory activities as they contained high polyphenolic contents. The polyphenolic compounds may inhibit NF-κB activation by inhibiting the activity of a molecule that activates NF-κB signaling or directly inhibits DNA binding of the NF-κB.[32] Previous studies on the anti-inflammatory of *Artemisia* species were stated. Testing extract of *A. annua*,[33] A. *fukudo*,[34] and *A. montana*[35] on murine RAW 264.7 macrophage cells showed anti-inflammatory activity by inhibiting LPS-induced nitric oxide and prostaglandin E$_2$ productions.

Our *A. lactiflora* extract might also have those similar anti-inflammatory activities.

This study only investigated anti-inflammatory activities of *A. lactiflora* extract and fractions in vitro using human innate immune cells model. Separation and study of phytochemicals by chromatography and mass spectrometry may provide details on its properties and benefit activities. Additionally, studies on cyclooxygenase and prostaglandin inhibitions as well as transcription factor translocation may explain molecular mechanisms underlying the anti-inflammatory activities of the *A. lactiflora* extract. Using *A. lactiflora* as an alternative medicine may help relieve inflammation, but we recommend consuming fresh leaves as part of food recipes to avoid toxicity to liver and kidney.

Conclusions

A. lactiflora has high phenolic and flavonoid contents and antioxidant activities. This study firstly reported anti-inflammatory activities of *A. lactiflora* in inhibiting the NF-κB-dependent signaling pathway, down-regulating expression of pro-inflammatory genes, and decreasing secretion of pro-inflammatory cytokines. It may be an alternative remedy to prevent respiratory tract inflammation due to air pollution from charcoal pyrolysis smoke.

Author Contributions

Research concept and design: NK, KB, MC
Collection and/or assembly of data: NK, KC, KN, NN, MC
Data analysis and interpretation: NK, NN, KY, MC
Writing the article: NK, KY, MC
Critical revision of the article: NK, KY, MC
Final approval of the article: NK, KY, MC

Ethical Approval

Methods and protocols involving human samples were approved by the Human Research Ethics Committee of Walailak University, project number: WU-EC-AH-2-129-63.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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