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Research article

Ethanolic extract of *Parkia speciosa* pods exhibits antioxidant and anti-inflammatory properties in lipopolysaccharide-induced murine macrophages by inhibiting the p38 MAPK pathway

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ABSTRACT

Background: Parkia speciosa (PS) is commonly used in Southeast Asian cuisine and traditional medicine to treat diabetes, hypertension, dermatitis, and kidney diseases. PS has emerged as a subject of interest because of its potential antioxidation and anti-inflammatory properties. However, despite its historically long and wide usage, a comprehensive investigation of these properties in PS pods (PSp) have not been conducted.

Aims of this study: This study aimed to identify the phytochemical compounds in the ethanolic extract of PSp collected from Southern Thailand and assess whether PSp exhibit antioxidant properties and mitigate inflammation in a lipopolysaccharide (LPS)-induced RAW264.7 model.

Materials and methods: The ethanolic extract of PSp was comprehensively analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC/MS) to identify its phytochemical constituents. To assess the antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) assays were performed, and cytotoxicity was evaluated using the MTT assay. The effect of PSp on reactive nitrogen and oxygen species (RNS and ROS) was determined using a nitric oxide (NO) assay, and its effect on pro-inflammatory cytokines was assessed using enzyme-linked immunosorbent assay (ELISA) and real-time quatitvative polymerase chain reaction (qPCR). Morphological changes following treatment were observed using a microscope. Western blot analysis was performed to quantify MAPK pathway expression.

Results: PSp contain polyphenols, phytosterols, triterpenes, oxaloacetic acid, and unsaturated fatty acids. PSp demonstrated high antioxidant potential in scavenging free radicals and exhibited no cytotoxic effects on macrophages. Moreover, PSp effectively reduced NO release and inhibited pro-inflammatory cytokines such as IL1-β, TNF-α, and IL-6. PSp treatment induced notable morphological changes in macrophages, characterized by an increase in cell size and the presence

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of intracellular vacuoles. In addition, Western blot analysis showed the selective suppressive effect of PSp on the p38-MAPK pathway.
Conclusion: PSp possess strong antioxidant and anti-inflammatory properties, making it a potential therapeutic agent for the treatment of inflammatory disorders.

1. Introduction

Inflammation is an innate immune response triggered by stress resulting from oxidative stress, tissue damage, or pathogen invasion. This reaction is a first-line defense mechanism that protects the body against injury and infection [1]. Acute inflammation manifests as a brief inflammatory response lasting a few days and is marked by the extravasation of plasma, erythrocytes, and leukocytes into the damaged tissue, whereas chronic inflammation persists longer and is characterized by the infiltration of macrophages and lymphocytes into the tissue [2]. Although inflammation is crucial for a healthy immune response, it also contributes to the development of various illnesses such as cancer, diabetes, arthritis, and cardiovascular disease [1]. Furthermore, it is generally believed that oxidative stress and chronic inflammation are the precipitating causes of aging and age-associated diseases [3,4].

Lipopolysaccharide (LPS) is a structural component of bacterial cells that comprises long polysaccharide chains covalently linked to lipids [5]. LPS can trigger the innate immune response via Toll-like receptors (TLRs), prompting macrophages to generate inflammatory mediators, including nitric oxide (NO) and other free radicals that can kill invading microbes [6]. Excessive levels of these enzymes can cause oxidative stress [4]. One of the downstream inflammatory pathways activated by LPS involves mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK. This pathway is implicated in the induction of the synthesis and release of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-1β [7], potentially leading to tissue inflammation [8].

Parkia speciosa (PS), also known as the stink bean or twisted cluster bean, is a plant species indigenous to Southeast Asia. Its seeds are commonly consumed as taste foods and are used in local traditional medicines for their medicinal properties, including curing diabetes [9] and cardiovascular diseases [10–12], and as antioxidants [13]. Several studies have identified bioactive compounds, particularly flavonoids and polyphenols, present in the seeds [14,15]. Pharmacological investigations have shown that these compounds extracted from the PS seeds affect human umbilical vein endothelial cells by decreasing the expressions of enzymes involved in the production of inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and vascular cell adhesion protein-1 (VCAM-1) [12]. Although the seeds of this plant are highly valued and widely consumed in food and traditional medicine, empty pods are generally discarded as waste. We hypothesized that the empty pods of PS (PSp) also contain chemicals similar to those in the valuable seeds, and that they have anti-oxidation and anti-inflammation properties, which require investigation and validation. Therefore, the present study aimed to determine the chemical composition, the antioxidation properties and investigate the anti-inflammatory effects and mechanism of action of PSp in LPS-induced RAW264.7 macrophage cells.

2. Materials and methods

2.1. PSp preparation and extraction

PS specimens were harvested from Chumphon, Thailand (GPS coordinate: 10°00′44″N 99°04′58″E) and identified by Dr. Narin Changkluengmao. A voucher specimen (No. PS-P-2023-001) were deposited in the Herbarium of the Food Bioactive Compounds Research Unit, Faculty of Allied Health Sciences, Burapha University, Chonburi, Thailand. The name was checked using World Flora Online, http://www.worldfloraonline.org, accessed on: April 18, 2023. The PS pods (PSp) were cleaned, deseeded, sliced, and dried before being ground into a powder using a grinder. The pod powder was extracted using the maceration method and soaked in absolute ethanol for 14 d. Subsequently, the ethanol-soluble phase was collected and centrifuged at 10,000 g for 30 min. The extract was then evaporated using an evaporator at 50 °C. The yield of the extract was 11.26 %. The extract was stored at −20 °C. The PSp stock solution was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) at a concentration of 10 mg/mL for further experiments.

2.2. Phytochemical profiling of PSp extract

The phytochemical profile of the extract was investigated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS). Non-targeted analysis of the phytochemicals in the PSp extract was performed using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) on a TripleTOF 6600 plus mass spectrometer (AB Sciex, USA) coupled with a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA). UHPLC was performed with Gradient program by using AcclaimTM RSLC 120C18 column (100 mm × 2.1 mm, 2.2 μm particle size, pore size 120 Å, Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 0.3 mL/min. The column was maintained at 40 °C. A gradient run was performed using two mobile phases: (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile (B). The method was set as follows: 0.00–1.00 min, 5 % B; 1.00–21.00 min, 5–95 % B; 21.00–25 min, 95 % B; and 25.10–30.00 min, 5 % B. The total run time of this method was 30 min/injection, and the injection volume was 2 μL.

Full-scan MS and MS/MS data were simultaneously gathered using information-dependent acquisition (IDA) under the following conditions: ion source gas 1 was set to 50 psi and ion source gas 2 was set to 60 psi. The gases used in the LC-MS analysis were nitrogen

as the nebulizer and collision gas. The ion source temperature was set at 500 °C. The ion spray voltage was set to 4.5 kV in the negative mode. Data were collected in the negative ionization mode by scanning over a mass range of 100–2000 *m/z*. In the MS/MS experiments, the mass range of the fragmentation products was set between 50 and 2000 *m/z* for the fragmentation products. The experiment was performed using a collision energy of −40 eV in negative mode. Phytochemical components were identified by comparing the MS spectra with the natural product high-resolution MS/MS Spectral Library on SCIEX OS (AB Sciex, USA) and the MS database of the National Institute of Standards and Technology (NIST).

GC-MS was performed on Agilent models 7890 B and 5977 B using a ZB-5MS UI column (30 m × 0.25 mm × 0.25 μm). The analysis method was set with a split ratio of 10:1 and an inlet temperature of 280 °C. Helium was used as a carrier gas at a flow rate of 1 mL/min. Initially, the oven temperature was programmed at 80 °C for the first 5 min, increasing a rate of 8 °C/min until 300 °C was reached and maintained for 16 min. The total run time was 45 min. The suspected chemical composition of the empty PS pod extract was compared with that in the W10N14.L database. Data were collected and identified for peaks with a quality of >90 %.

2.3. Free radical scavenging assays

The free radical-scavenging activities of PSp extracts were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) assays. In DPPH assay the PSp extract was diluted in ethanol at various concentrations (1.25–40 μg/mL). A 0.2 mM DPPH (EMD Millipore, Germany) solution was prepared by dissolving the chemical in absolute ethanol and then mixing with the designated concentrations of the extract. The mixtures were then incubated in the dark at room temperature for 20 min. Absorbance was measured at 518 nm. The radical scavenging activity was calculated using the following equation:

$$\text{DPPH \% radical scavenging activity} = \frac{\textit{Absorbance of control} - \textit{Absorbance of sample}}{\textit{Absorbance of control}} \times 100$$

An ABTS assay was conducted to assess the scavenging of radical cations. The ABTS solution was prepared by mixing 7 mM ABTS (EMD Millipore, Germany) with 2.45 mM potassium persulfate ($K_2S_2O_8$). Next, the solution was stored in the dark at room temperature for 16 h to facilitate the generation of free radicals. The ABTS stock solution was diluted with distilled water to obtain a working solution. To determine the scavenging activity, the ABTS working solution was mixed with varying concentrations of PSp (1.25–40 μg/mL) in a 96-well plate and incubated at room temperature for 6 min. Following incubation, the absorbance was measured at 734 nm. Scavenging activity was calculated using the following equation:

$$\text{ABTS \% radical scavenging activity} = \frac{\textit{Absorbance of control} - \textit{Absorbance of sample}}{\textit{Absorbance of control}} \times 100$$

Trolox (Sigma-Aldrich) was used as a reference. The half-maximal effective concentration (EC_{50}) was calculated based on the percentage inhibition curves. The results were expressed as milligrams of trolox equivalent antioxidant capacity (TEAC) per gram of extract.

2.4. Cell line and cell culture

The murine macrophage cell line RAW264.7 (*RRID: CVCL* 0493), was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA; lot number: 70012232). The cell line was authenticated by the ATCC on March 28, 2018, using a cytochrome oxidase I (COI) assay to confirm its species as mouse (*Mus musculus*). The cells were verified to be mycoplasma-free using Hoechst staining, agar culture, and a polymerase chain reaction (PCR)-based assay. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1 % penicillin/streptomycin. The cells were incubated in a humidified incubator with 5 % CO_2 at 37 °C.

2.5. Cell viability assay

Cells were seeded at a density of 1×10^5 cells/well in 96-well plates and incubated for 24 h. Subsequently, serial dilutions of PSp were prepared in complete cell culture medium. The cells were treated with various concentrations of PSp for 24 h. At the indicated time, 0.5 mg/mL tetrazolium salt (MTT) was added and the cells were incubated for 4 h. Following the incubation period, the formazan crystals were dissolved using 100 μL of dimethyl sulfoxide (DMSO; Fisher Scientific, Belgium). The absorbance of the samples was measured at 570 nm using a microplate reader (ThermoFisher). The percentage of viable cells was calculated and compared with that of the negative control.

2.6. NO generation

To induce NO production, LPS derived from *Escherichia coli* O111:B4 (Sigma-Aldrich) was added RAW264.7 cells at a concentration of 0.1 μg/mL. Subsequently, the cells were exposed to designated concentrations of PSp extract for 24 h. A volume of 100 μL from the supernatant of each experimental group was collected and incubated with 100 μL Griess Reagent. Absorbance was measured at 540 nm using a microplate reader (ThermoFisher).

2.7. Enzyme-linked immunosorbent assay (ELISA)

IL1-β, IL-6, and TNF-α ELISA (Millipore, MO, USA) assays were used to determine the anti-inflammatory effects of PSp on pro-inflammatory cytokines production. Cell supernatants from each group were collected 24 h after exposure to LPS and the designated concentrations of the extract. Detection was performed according to the manufacturer's instructions. Briefly, 100 μL of the sample was added into the primary antibody coated plate and incubated for 2.5 h at room temperature. The plates were washed and incubated with biotin for 1 h. The plate was washed again and incubated with horseradish peroxidase-streptavidin for 45 min. Color was developed using a TMB substrate. The absorbance was measured at 450 nm using a microplate reader (ThermoFisher).

2.8. RNA isolation, reverse transcription, and real-time quantitative (q)PCR

The cells were collected after 24-h incubation with PSp extract and LPS. Total RNA was isolated using the (GeneAid, Taiwan). RNA concentration was measured using a NanoDrop spectrophotometer. cDNA was synthesized using an iScript™ cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. Quantification was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The PCR conditions were 95 °C for 1 min, 95 °C for 15 s, and 60 °C for 30 s for a total of 40 cycles. The mRNA expression levels of *iNOS, IL-1β, IL-6,* and *TNF-α* were normalized to that of GAPDH. The $2^{-\Delta\Delta Ct}$ method was used to calculate the data, and the primer sequences are listed in Table 1.

2.9. Western blot analysis

Cells were seeded in six-well plates at a density of 6×10^5 cells/well. Following overnight incubation, the cells were exposed to LPS and specific concentrations of PS for 24 h. Subsequently, cells were harvested and lysed using a nuclear extraction kit (Millipore, Germany). Protein concentrations were measured using bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific). A protein quantity of 10 μg/mL from each group was subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Amersham) using a wet electrophoretic cell. The membranes were blocked with 5 % bovine serum albumin (BSA) buffer for 1 h at room temperature. Following blocking, the membranes were incubated overnight at 4 °C in a shaking incubator with primary antibodies sourced from the MAPK family antibody sampler kit (Cell Signaling). At the indicated time points, the membranes were washed three times with 0.1 % Tween-20 in Tris-buffered saline (TBST). The membranes were then incubated with secondary antibodies diluted in blocking solution. The proteins of interest were visualized using enhanced chemiluminescence (ECL) substrate (Thermo Scientific). Band intensities were quantified using ImageJ software and the values were normalized to those of actin.

2.10. Statistical analysis

GraphPad Prism 9.5.0 (GraphPad Software, Inc., USA) was used for the statistical analyses. The data are expressed as means ± standard deviation (SD) of three independent experiments. One-way analysis of variance (ANOVA) was used followed by Turkey's post hoc test. Statistical significance was set at $*p < 0.05$ and assessed among the groups.

3. Results

3.1. Phytochemical compositions in PSp extract

As shown in Fig. 1, GC-MS analysis revealed 20 compounds in the PSp extract. Table 2 lists some of the most important bioactive compounds, including 1,2,3-benzenetriol (60.48 %), 1,3,5-benzenetriol (5.85 %), 2-hexadecanoyl glycerol (3.20 %), 24(Z)-methyl-25-homocholesterol (2.37 %), hexadecenoic acid (1.73 %), vitamin E (0.84 %), α-amyrin (0.80 %), and ethyl palmitate (1.73 %). Four compounds showed >99 % in the negative-mode LC-MS/MS data library. These compounds include oxaloacetic acid, epigallocatechin gallate, hyperin, and quercitrin (Table 3). Furthermore, only two compounds, 4-Methoxytriphenylmethyl and piperine, were detected

Table 1
Primer sequences used in this study.

Genes	Primers (5' → 3')	Lengths (bp)
iNOS	F: GCTCGCTTTGCCACGGACGA	20
	R: AAGGCAGCGGGCACATGCAA	20
TNF-a	F: CCCTCCTGGCCAACGGCATG	20
	R: TCGGGGCAGCCTTGTCCCT	20
IL-6	F: AGACAAAGCCAGAGTCCTTCAGAGA	25
	R: GCCACTCCTTCTGTGACTCCAGC	23
IL1-b	F: GGGCCTCAAAGGAAAGAATC	20
	R: TACCAGTTGGGGAACTCTGC	20
GAPDH	F: ACCCCAGCAAGGACACTGAGCAAG	24
	R: GGCCCCTCCTGTTATTATGGGGGT	24

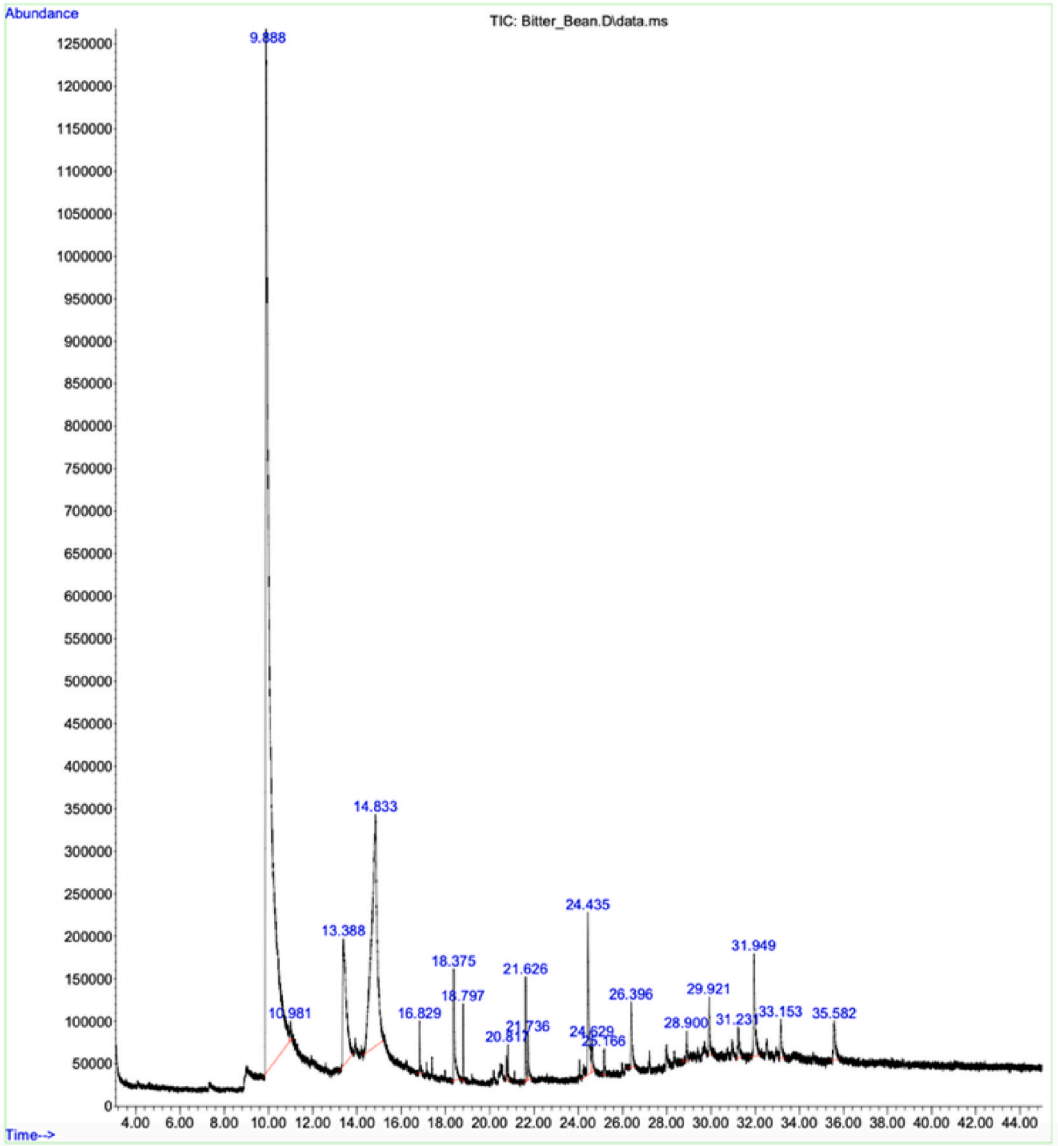

Fig. 1. GC-MS chromatogram of empty PSp extract.

Table 2
Major chemical compositions present in PSp ethanolic extract detected using GC-MS.

Retention time (min)	%Area	Chemical name
9.888	60.48	1,2,3-Benzenetriol
13.388	5.85	1,3,5-Benzenetriol
18.375	1.73	Hexadecenoic acid
18.797	0.55	Ethyl palmitate
24.435	3.20	2-Hexadecanoyl glycerol
29.921	0.84	Vitamin E
31.949	2.37	24(Z)-Methyl-25-homocholesterol
33.153	0.80	α-Amyrin

Table 3
Compounds present in PSp ethanolic extract detected using negative ion mode LC-MS/MS and identified by comparing their retention times and MS data with those of reference compounds.

Compounds	Retention time (min)	$[M-H]^-(m/z)$	Library score
Oxaloacetic acid	0.91	131.0359	100
Epigallocatechin gallate	5.60	457.0769	99.5
Hyperin	6.75	463.0885	99.5
Quercetin	7.42	447.0941	99.1

Table 4
Compounds present in PSp ethanolic extract detected using positive ion mode of LC-MS/MS and identified by comparing their retention times and MS data with those of reference compounds.

Compounds	Retention time (min)	$[M+H]^+$ (m/z)	Library score
4-Methoxytriphenylmethyl	7.40	273.0764	100
Piperine	13.21	286.1443	99.7

in the positive-mode LC-MS/MS (Table 4). These data demonstrate that PSp contains abundant phytochemical compounds that potentially contribute to various biological activities.

3.2. Antioxidant activities of PSp extract

The antioxidant activity of the PSp extract was investigated using DPPH and ABTS assays. The DPPH radical scavenging activity of the PSp extract is shown in Table 5. The extract exhibited notable antioxidant activity against DPPH radicals in a concentration-dependent manner. The percentage of DPPH radical scavenging activity ranged from 14.85 ± 1.97 to 91.57 ± 0.09, with an EC_{50} of 6.39 μg/mL (Fig. 2A). Similarly, PSp extract exhibited varying percentages of ABTS radical scavenging activity, ranging from 36.77 ± 2.11 to 93.70 ± 0.12, with an EC_{50} of 2.13 μg/mL (Fig. 2B). Trolox, which was used as a reference antioxidant molecule, showed EC_{50} values of 4.87 and 2.03 μg/mL as determined using the DPPH and ABTS assays, respectively. The PSp extract revealed the TEAC values of 762.13 and 953.05 mg Trolox/g extract, calculated from the DPPH assay and ABTS assay, respectively. Collectively, these data indicate that PSp possesses high antioxidant potential for scavenging free radicals.

3.3. PSp exhibited no cytotoxicity

The MTT assay was performed to examine the cytotoxicity of PSp extract on RAW264.7 cells. As shown in Fig. 3A, a concentration of 200 μg/mL of PSp significantly decreased cell viability to 70.63 ± 1.48 %, whereas lower concentrations did not exhibit any cytotoxic effects. In addition, under LPS-stimulated conditions, the extract showed no cytotoxic effects at any concentration compared to the control (Fig. 3B).

3.4. PSp extract decreased LPS-induced NO production in murine macrophage cell line

LPS induces an inflammatory response in macrophages by triggering the release of NO and high levels of cytokines [16]. As shown in Fig. 4, a small amount of NO was detected in the negative control. In contrast, cells stimulated with 0.1 μg/mL LPS exhibited a maximum NO level of 28.35 μM. Consequently, the influence of PSp extract on NO generation was investigated in the presence of 0.1 μg/mL LPS. As illustrated in Fig. 4, PSp inhibited NO production in a concentration-dependent manner. Notably, PSp at concentrations of 100–200 μg/mL significantly attenuated NO release compared with the control. The IC_{50} of PSp were 96.69 μg/mL. Similarly, the concentration of iNOS was measured as shown in Fig. 4B. PSp at concentrations 50, 100, and 150 μg/mL significantly inhibited iNOS expression. Therefore, these concentrations were selected for subsequent investigations.

3.5. PSp extract inhibited IL1-β and IL-6 expression at both transcriptional and translational levels

To mimic a chronic inflammatory environment, macrophages secrete pro-inflammatory cytokines prior to LPS challenge. IL1-β, TNF-α, and IL-6, levels measured either directly in the supernatants or from the mRNA levels exhibited a significant increase following

Table 5
EC_{50} and Trolox equivalent values of PSp extract.

Samples	EC_{50} (μg/mL)		Trolox/extract (mg/g)	
	DPPH	ABTS	DPPH	ABTS
PS pod extract	6.39	2.13	762.13	953.05
Trolox	4.87	2.03	–	–

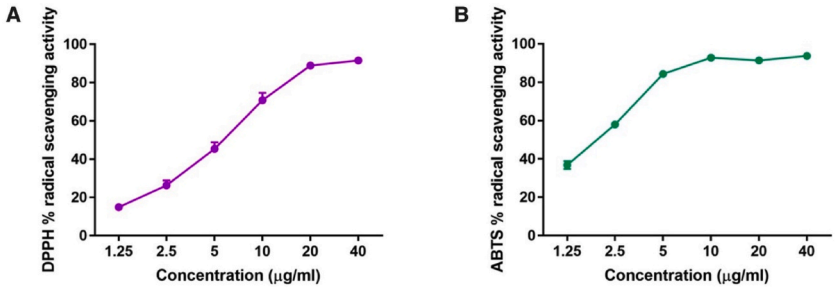

Fig. 2. Antioxidant activities of PSp extract. A) DPPH % radical scavenging activity of PSp extract. B) ABTS % radical scavenging activity of PSp extract. Each value in the graph is expressed as mean ± standard deviation (SD) (n = 3).

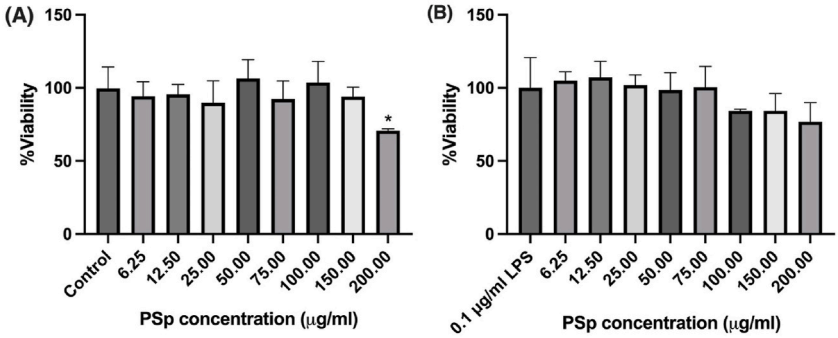

Fig. 3. Effect of PSp extract on viability of murine macrophages; RAW264.7 detection was performed using the MTT assay. (A) Cells were treated with extract (0–200 μg/mL) for 24 h. (B) Cells were treated with 0.1 μg/mL LPS and PSp (concentrations ranging 0–200 μg/mL) for 24 h. Values in the graphs are expressed as mean ± SD (n = 3). Statistical significant, denoted as $*p < 0.05$, was assessed among the specified groups.

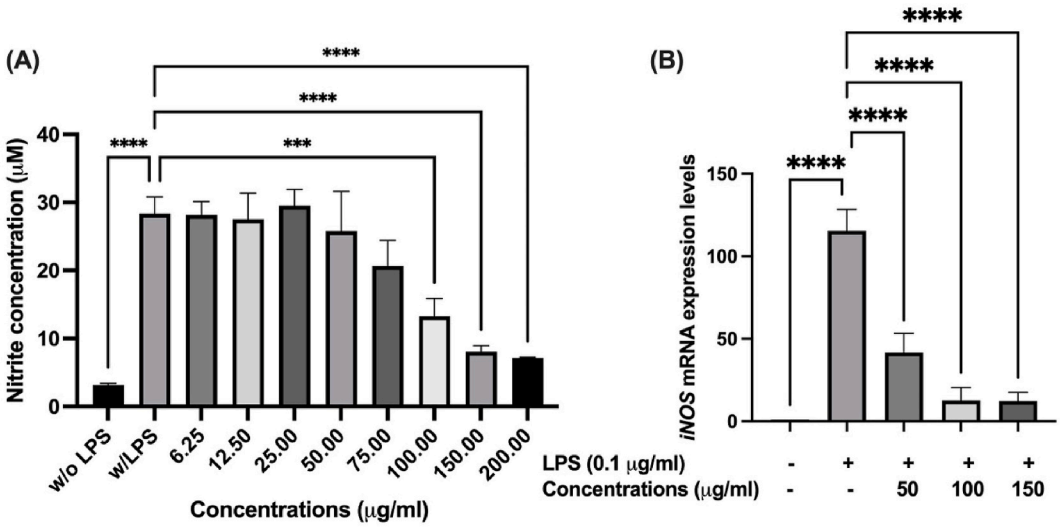

Fig. 4. Effect of PSp extract on NO production. (A) Murine macrophages were categorized into the negative control (not induced by LPS) and experimental groups treated with 0.1 μg/mL LPS in combination with varying concentrations of PSp extract (0–200 μg/mL). (B) Real-time PCR was performed to detect *iNOS* expression. Each data point is presented as mean ± SD (n = 3). Statistical significant, denoted as $*p < 0.05$, was assessed among the specified groups.

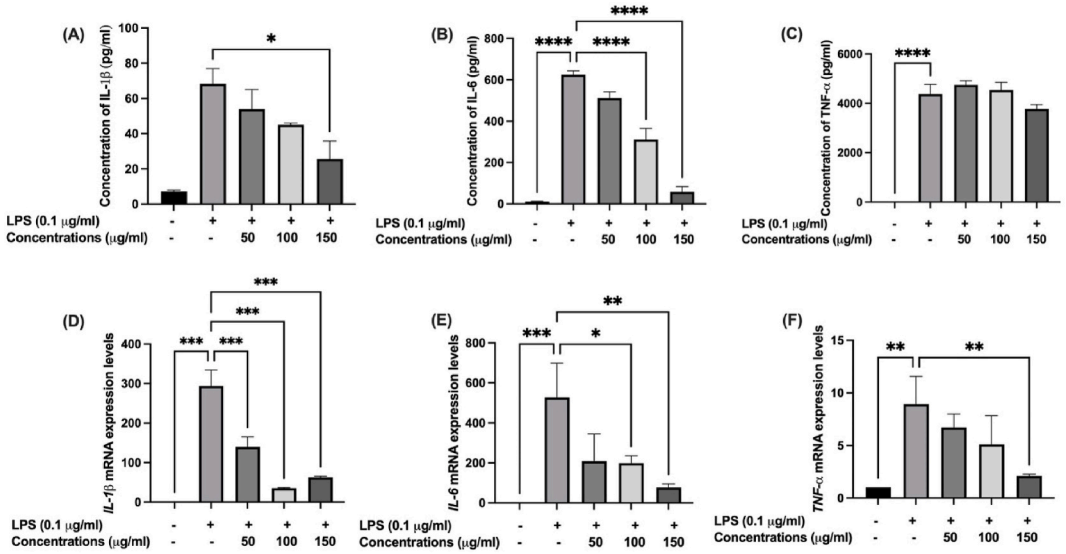

Fig. 5. Effects of PS on proinflammatory cytokine expression at transcriptional and translational levels. (A–C) ELISA were performed to examine IL1-β, IL-6, and TNF-α concentrations in cell supernatants. (D, E) Real-time PCR was performed to examine expression levels of mRNAs encoding IL1-β, IL-6, and TNF-α. *GADPH* mRNA expression was used as an internal reference. Each data point is presented as mean ± SD (n = 3). Statistical significant, denoted as $*p < 0.05$, was assessed among the specified groups.

exposure to LPS for 24 h (Fig. 5A and C). PSp treatment resulted in a notable decrease in the levels of these pro-inflammatory cytokines in a concentration-dependent manner (Fig. 5A–F). Both IL1-β and IL-6 exhibited significant decreases at both transcriptional and translational levels, whereas TNF-α exhibited a marked reduction only at the mRNA level. Collectively, these data demonstrate the potential of PS as an anti-inflammation agent that diminishes pro-inflammatory cytokine expression.

3.6. PSp extract altered morphology of LPS-induced macrophages

Morphological alterations in macrophages were observed concomitantly with their functional activation upon stimulation with LPS, as shown in Fig. 6. In the absence of stimulation, the cells exhibited a rounded morphology with diminutive cytoplasm and multiple pseudopodia. Exposure to 0.1 μg/mL LPS resulted in the formation of vacuolar structures within the cytoplasm of RAW264.7 cells. Interestingly, treatment with PSp caused a discernible augmentation in cell size, accompanied by an increase in the number of vacuoles within the cytoplasm. These findings suggest that PSp interferes with the vacuolization process in macrophages.

3.7. PSp inhibited the p38 MAPK pathway

The MAPK pathway is recognized as one of the pivotal pathways involved in modulating the inflammatory response triggered by LPS activation of TLRs. Consequently, Western blot analysis was conducted to investigate the effect of PSp extract on the activation of p38 MAPK, JNK, and ERK1/2. As shown in Fig. 7A, following a 1-h treatment, LPS significantly elevated the levels of phosphorylated p38 and JNK proteins compared to the untreated control group. At all concentrations demonstrated selectively inhibited LPS-induced phosphorylation of p38 proteins, whereas phosphorylated JNK and p-ERK proteins remained unaffected. These findings suggest that PS elicits an anti-inflammatory effect by attenuating the p38-MAPK signaling pathway.

4. Discussion

In Southeast Asia, the seeds, leaves, and roots of PS have long been used as culinary ingredients with various therapeutic applications [9], whereas PS pods are considered residual components lacking in value and specific usefulness. Previous studies have demonstrated that the seed of PS contains saponins, alkaloids, flavonoids, polyphenols, and terpenoids [17]. Although a previous study showed that the alcohol extract of PSp primarily contains flavonoids and phenolics [18], our LC-MS/MS and GC-MS analyses revealed that PSp also harbored phytochemicals similar to those found in its seeds, including phytosterols, triterpenes, oxaloacetic acid, and unsaturated fatty acids. The compounds such as 1,2,3-benzenetriol (pyrogallol), hexadecenoic acid (palmitoleic acid), vitamin E, a-Amyrin, epigallocatechin gallate, quercetin, and piperine possess notable antioxidant and anti-inflammatory properties [19–21]. Importantly, we showed that PSp contains chemicals similar to those found in PS seeds and that they have the potential to inhibit inflammation.

Inflammation is a fundamental protective mechanism that is triggered in response to pathogen invasion and tissue injury. To assess the potential anti-inflammatory effects of the PSp extract, we stimulated murine macrophages with LPS to mitigate chronic

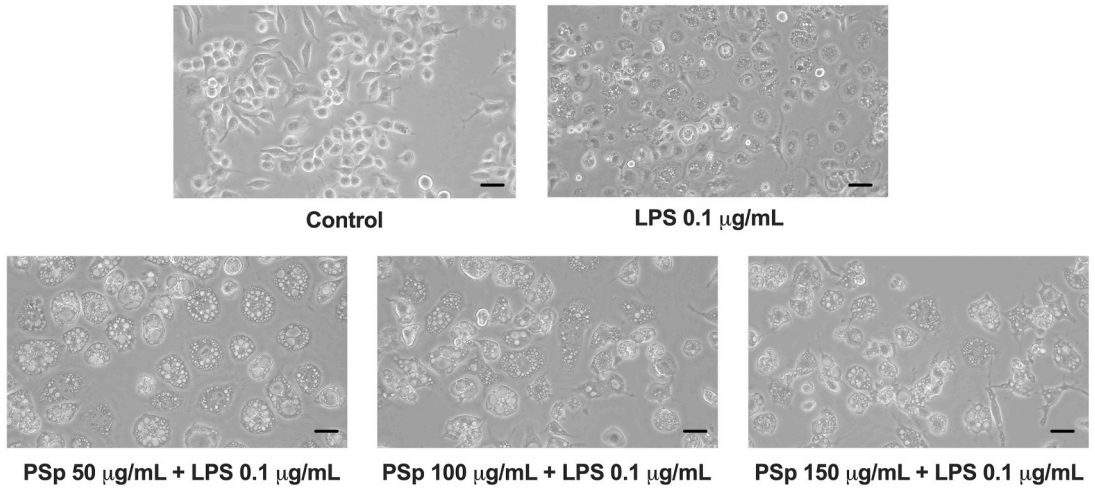

Fig. 6. Effect of PSp extract on the morphology of LPS-induced macrophages. Cells were treated with the designated conditions for 24 h. Cell morphology was observed using phase contrast microscopy (magnification, 40 ×). Scale bar, 100 μm.

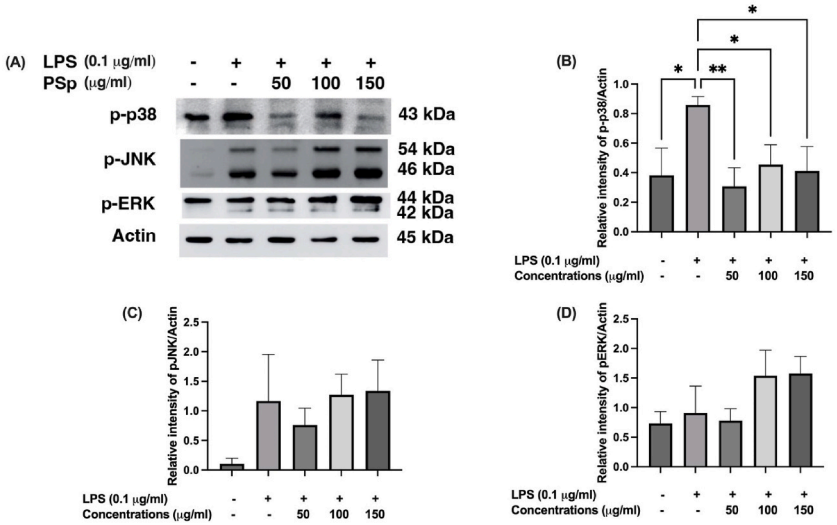

Fig. 7. Effect of PSp extract on MAPK induced by LPS. (A) Western blot analysis was performed to assess p-p38, p-JNK, and p-ERK protein levels. Actin was used as an internal reference. Densitometric data represent the means of three independent experiments assessing (B) p-p38, (C) p-JNK, and (D) p-ERK levels. Statistical significant, denoted as $^{*}p < 0.05$, was assessed among the specified groups.

inflammation. In this cellular model, a cascade of inflammatory mediators, including ROS, IL1-β, TNF-α, and IL-6, are released [22]. By measuring these proinflammatory cytokines, we showed that PSp contained chemicals extractable by ethanol that strongly inhibited inflammation. This property is crucial for PSp to be used for therapeutic purposes. During the aging process, increasing levels of ROS and RNS are generated from several metabolically active sources, including dysfunctional mitochondria and cytochrome P450, which are involved in the catabolism of drugs and exogenous compounds; NADPH oxidase; oxidative stress in the endoplasmic reticulum; various NOS enzymes; and several other oxidoreductase enzyme systems [3]. Excessive levels of ROS and RNS can oxidize various biological components, including proteins, lipids, and nucleic acids, thereby resulting in altered molecules recognizable as non-self by the immune system. This initiates inflammation and a subsequent adaptive immune response that may cause cellular and tissue damage, leading to age-associated diseases [4]. Therefore, balancing ROS levels has been proposed to protect against cellular damage and attenuate inflammatory responses, consequently preventing age-associated diseases [23]. Our results obtained from antioxidant assays revealed that PSp exhibited potent scavenging activity against oxidative stress and suppressed iNOS expression, which generates NO that may give rise to other RNS. Similarly, previous investigations of the ethyl acetate and ethanolic fractions of *P. speciosa* pods showed that the pods contain phenolics, flavonoids, and terpenes, which exhibit antioxidant activity by suppressing intracellular ROS, NO, and iNOS in angiotensin II-induced cardiac hypertrophy [10,12]. PSp also inhibited the expression of inflammatory mediators at both the transcriptional and translational levels. Among these mediators, IL-6 was the most negatively affected by the

treatment with PSp, followed by IL-1β and TNF-α. Furthermore, the MTT assay showed that PSp had no significant impact on cytotoxicity at any concentration, indicating that the observed anti-inflammatory effects were attributable to diminished cell viability.

Mitogen-activated protein kinases (MAPKs), which include extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK, play a vital role in cellular responses to various stimuli, including LPS-induced inflammatory response [24]. ERK, JNK, and p38 MAPK activation influences inflammation by modulating the expression of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α [25,26]. LPS activation through TLR4/p38 MAPK signaling in macrophages accelerates cytoskeletal rearrangement, leading to cell elongation and flattening [27]. Similarly, in this study, exposure to LPS caused macrophages to undergo morphological changes, characterized by a transition to a round shape, with multiple pseudopodia and small vacuoles. Moreover, alterations in cellular morphology, including increased cell size and the presence of larger intracellular vacuoles, were observed in the PSp treatment group. The formation of large vacuoles represents a distinctive pattern associated with inhibition of p38 MAPK as opposed to other inflammatory pathways [28]. Consistent with this finding, Western blot analysis indicated that PSp extract selectively inhibited phosphorylated p38 MAPK expression. Collectively, these data indicate that the PSp extract exerts antioxidation and anti-inflammation effects, suggesting that PSp is a potential therapeutic agent for inflammatory disorders and age-associated diseases.

CRediT authorship contribution statement

Tepparit Samrit: Writing – original draft, Methodology, Investigation. **Narin Changklungmao:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Kant Sangpairoj:** Methodology, Investigation. **Aticha Buddawong:** Methodology. **Pornanan Kueakhai:** Writing – review & editing, Resources. **Kititpong Chuanboon:** Methodology, Investigation. **Prasert Sobhon:** Writing – review & editing, Supervision, Conceptualization. **Kanta Pranweerapaiboon:** Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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