Arch Biol Sci. 2024;76(3):335-343 https://doi.org/10.2298/ABS240704025K

Ultrasound-assisted extraction of peanut shell by-product: chemical properties, antioxidant, and anti-inflammatory effects

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Received: July 4, 2024; **Revised**: July 16, 2024; **Accepted**: July 18, 2024; **Published online**: August 23, 2024

Abstract: Peanut shell by-products have been explored for their pharmacological potential, particularly through applications developed from their utilization. This study aimed to investigate the effects of peanut shell extract (UPE) obtained via ultrasound-assisted extraction (UAE) on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. High-performance liquid chromatography analysis revealed elevated levels of luteolin in the ultrasound-extracted peanut shell extract (UPE). UPE demonstrated significant *in vitro* antioxidant activity, as evidenced by its ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals. The anti-inflammatory effects of UPE were assessed using the nitric oxide (NO) Griess assay, prostaglandin E2 (PGE2), and interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA). Western blot analysis and reverse transcription polymerase chain reaction (RT-PCR) were used to evaluate the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). UPE significantly reduced NO, PGE2, and IL-6 levels in LPS-treated RAW 264.7 cells, suggesting potent anti-inflammatory properties. Furthermore, UPE downregulated the expression of iNOS and COX-2, thereby suppressing NO and PGE2 production. These findings indicate that peanut shell extracts obtained through UAE have therapeutic potential due to their enhanced antioxidant and anti-inflammatory effects, likely attributed to increased levels of luteolin.

Keywords: peanut shell, ultrasound-assisted extraction, luteolin, antioxidant, anti-inflammatory

INTRODUCTION

Inflammation is a complex biological response to tissue damage induced by various harmful stimuli, including toxins and pathogens [1]. It can lead to excessive exposure to inflammatory mediators, resulting in cellular damage and tissue necrosis. Macrophages are immune cells that are essential in modulating innate immunity, preventing antigens, and producing cytokines that activate inflammation [2]. Lipopolysaccharide (LPS) stimulation enables macrophages to recognize stimulation through receptors, including Toll-like receptor (TLRs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen-activated protein kinases (MAPK) [3-5]. This process also promotes the generation of nitric oxide (NO) and prostaglandin E2 (PGE_2), which induce inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [6]. The regulation of these inflammatory mediators is a key target for treating inflammation.

Peanut (*Arachis hypogaea* L.) is an agricultural crop grown in the warm climates of Asia [7]. Peanuts contain approximately 30% crude protein on a dry weight basis, second only to soybean [8]. They also contain phenolic compounds, vitamins, resveratrol, luteolin, procyanidins, and essential amino acids. Recently, peanuts have attracted increasing interest because of their health benefits [9]. Because of the consumption of peanut products, by-products are generated during agricultural processing. Moreover, the functional properties of peanuts have led to the development of high-value applications for their by-products [10]. Despite abundant nutrient compounds, peanut shells remain

underutilized as a valuable resource in functional products. Peanut shells have been reported to exhibit antioxidant and anticancer effects, varying by cultivar and extraction solvent. These effects are linked to the content of luteolin and eriodictyol [11]. A previous study used diverse extraction techniques to examine the effects of peanut shells in improving bioactivation by extracting polyphenols from natural products using fast and appropriate UAE, a cost-effective method due to the reduction in the amount of solvent used [12].

Luteolin is one of the flavone compounds present in various plant species such as fruits and vegetables. Plants rich in luteolin have long been utilized in traditional medicines of China, Japan, and Korea [13]. Luteolin possesses beneficial biological properties, including antioxidant, antibacterial, anti-inflammatory, and anti-cancer effects [14]. It possesses potent reactive oxygen species (ROS) activity [15]. Another study revealed that luteolin reduces inflammation by inhibiting the expression of pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The reported mechanism directly involves the downregulation of interleukins 1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α) in cellular models [16].

UAE has been used to extract bioactive compounds [17]. Ultrasound at a frequency of 20 kHz or more helps improve the solvent's ability to penetrate cells by disrupting the cell wall [18]. Previous studies reported that UAE could improve the functional phytochemical properties of soybean, rosemary, and peppers [19-21]. However, the effects of luteolin from peanut shell extracts obtained using UAE on inflammatory responses have not yet been reported. Therefore, the present study investigated the effects of luteolin from peanut shell extract obtained using UAE on LPS-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Extraction of peanut shell extract using ultrasound-assisted extraction (UAE)

Peanut shells were obtained from Udounni Company (Jeju, Korea) as a by-product of peanut seeds. They were manually washed and dried at 50° for three days before extraction. Dried peanut shells were sonicated with 70% ethanol using an ultrasonic extraction system (Powersonic 420, Korea) at a working frequency of 40 kHz for 24 h. The extract was filtered using filter paper, and the solvent was removed by decompression. The sample was frozen and stored at -20° until use.

High-performance liquid chromatography (HPLC) analysis

Luteolin identification was conducted using a Shimadzu HPLC (Kyoto, Japan) system equipped with a Shimpack GIS C18 column (4.6 mm × 250 mm, 5 μm) and a photodiode array (PDA) detector. The mobile phase comprised solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile). The flow rate of the mobile phase was 1 mL/min. Luteolin in the UPE was identified by comparing the retention times and UV spectra of samples analyzed under the same chromatographic conditions.

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

DPPH radical scavenging activity was determined by the methods of Blois et al. [22] with some modifications. Sixty μL of DPPH solution and 120 μL of extract at different concentrations were added to each sample. The mixture was incubated for 15 min. Absorbance was measured at 517 nm using a microplate reader.

Determination of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) free radical scavenging assay

The antioxidant activity of UPE was assessed using the $ABTS^+$ assay, which was performed following an improved protocol from our previous study [23]. Five mL of a 7-mM ABTS solution and 88 μL of 140 mM $K_2S_2O_8$ were mixed for 14 h; 3 mL of ABTS was added with 150 μL of sample. Following the reaction, the absorbance of the reactant was measured at 734 nm.

Cell culture

The RAW 264.7 macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). These cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal

bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a 5% CO_2 incubator. The cells were subcultured once every two days.

Cell viability assay

The cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h. Next, they were treated with varying UPE concentrations and incubated overnight. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were added to MTT solution for 4 h and dissolved in dimethyl sulfoxide (DMSO). The absorbance of cells was measured at 540 nm using a microplate reader.

Nitrite determination

To measure the inhibition of NO production, nitrite accumulation in the culture medium was determined by the method of Green et al. [24]; the cells were cultured in a six-well plate (3×10^5 cells/well) for 24 h and then pretreated with LPS for 2 h. The incubated cells were treated with different UPE concentrations for 24 h. Subsequently, 100 μL of samples was mixed with 100 μL of Griess reagent incubated at room temperature for 10 min. The absorbance of samples was measured at 540 nm.

Evaluation of interleukin (IL)-6 and PGE_2 expression

The expression of IL-6 and PGE_2 in macrophages was quantified using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions. The absorbance of treated samples was measured at 450 nm using a plate reader.

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using TRIzol (Invitrogen, CA, USA) following the manufacturer's instructions. One μg of RNA was reverse transcribed using 1 mM dNTPs, oligo dT primers, 5× Green GoTaq Flexi Buffer, and Taq DNA polymerase (Promega, Madison, WI, USA). GAPDH was used to normalize gene expression levels. The primer sequences of genes are as follows: iNOS (reverse) GCTGTGTGTCACAGAAGTCTCGAACTC, (forward) AATGGCAACATCAGGTCGGCCATCACT; COX-2 (reverse) ATGGTCAGTAGACTTTTACA, (forward) GGAGAGACTATCAAGATAGT.

Western blot analysis

The cells were pretreated with UPE (2.5, 5, and 10 μg/mL) following induction with UVB irradiation (20 mJ/cm^2). The cells were harvested and lysed using radioimmunoprecipitation assay buffer (RIPA) buffer. After protein lysis, a concentration gradient of bicinchoninic acid was used for standardization. After separation on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAG) the proteins were transferred to a polyvinylidene difluoride membrane. After blocking with skim milk for 1 h, the membranes were incubated overnight with primary antibodies to iNOS, COX-2, and β-actin. The blots were washed with Tris-buffered saline with 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized using enhanced chemiluminescence reagents and quantified using the ChemiDoc Imaging System.

Statistical analysis

All experiments were performed in triplicate. Data are presented as the means±standard deviation. Statistical significance was considered at $P<0.05$. All statistical analyses were conducted using SPSS version 20 (IBM Corp., Armonk, NY, USA).

RESULTS

Composition of luteolin of UPE

HPLC measured the profile of compounds from UPE with mobile phase systems using a PDA detector. Luteolin was confirmed using 345 nm UV irradiation with a C18 column for 22 min (Table 1). The peak retention time of the index component of luteolin matched the peak retention time of the index component of UPE, confirming the specificity through their spectra. In addition, as the ultrasound duration increased, the amount of luteolin in the peanut shell

extract also increased. The findings indicated that peanut shell extracts had significantly increased luteolin expression by 1.8-fold.

Table 1. Results of high-performance liquid chromatography showing the luteolin contents of 70% ethanol and peanut shell using ultrasound-assisted extraction.

Extract	Solvent	Luteolin (%)
Peanut shell	70% EtOH extraction	1.96±0.01
	Ultrasound-assisted extraction	3.57±0.01

The results are presented as the mean±standard deviation (n = 3).

In vitro antioxidant activity of UPE

The DPPH and ABTS$^+$ radical scavenging activities of UPE were investigated. The antioxidant activity of UPE was compared with that of vitamin C used as a positive control. As shown in Fig. 1, the DPPH and ABTS$^+$ assays revealed that the radical scavenging activity of UPE increased in a dose-dependent manner. Treatment with 50 μg/mL of UPE exhibited strong radical scavenging activity compared with the standard.

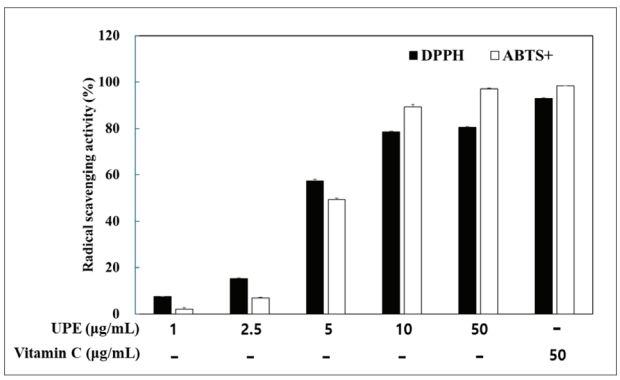

Fig. 1. Electron-donating and ABTS radical scavenging activity of peanut shell extracts using ultrasound-assisted extraction. UPE – ultrasound peanut shell extract. All data results are presented as the mean±standard deviation of three independent measurements.

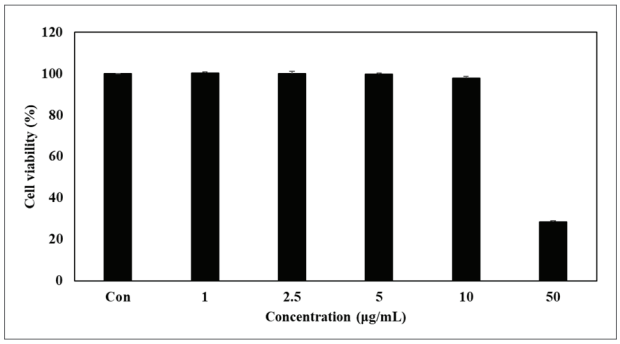

Fig. 2. Viability of RAW 264.7 cells treated with UPE. Con – control, extract-free group of RAW 264.7 cells; UPE – ultrasound peanut shell extract. After RAW 264.7 cells were cultured for one day in a 96-well plate, they were treated with 1, 2.5, 5, 10, and 50 μg/mL UPE for 24 h. Cell viability was measured using the MTT assay. The results are presented as the mean±standard deviation of three independent measurements.

Cell viability in RAW 264.7 cells

The cytotoxicity of UPE was evaluated in RAW 264.7 cells. The sample concentration was determined by analyzing cell viability in the presence of different UPE concentrations (0-50 μg/mL). Cytotoxicity was maintained at over 90% when the cells were treated with 10 μg/mL doses of sample (Fig. 2). Thus, non-cytotoxic concentrations were used to examine the efficacy of UPE.

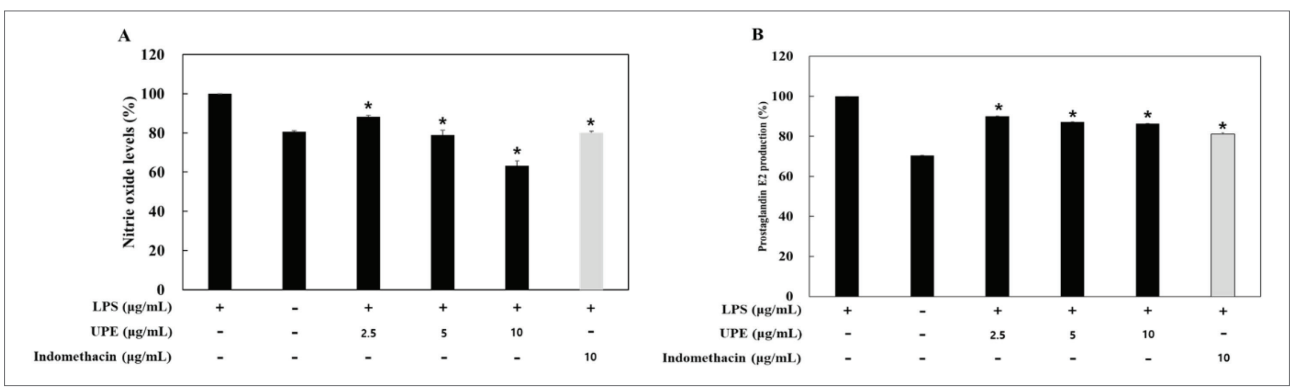

Fig. 3. Inhibitory effects of UPE on NO production in LPS-stimulated RAW 264.7 cells. **A** – NO production was determined in RAW 264.7 cells treated with UPE and indomethacin. **B** – PGE_2 production was determined in RAW 264.7 cells treated with UPE and indomethacin. UPE – ultrasound peanut shell extract. After RAW 264.7 cells were cultured for one day in a six-well plate (3×10^5 cells/well), they were treated with LPS (10 μg/mL) for 2 h. The cells were treated with UPE and indomethacin and measured 18 h later. Results are presented as the mean±standard deviation from three measurements (*P<0.05 vs. the LPS alone treatment group).

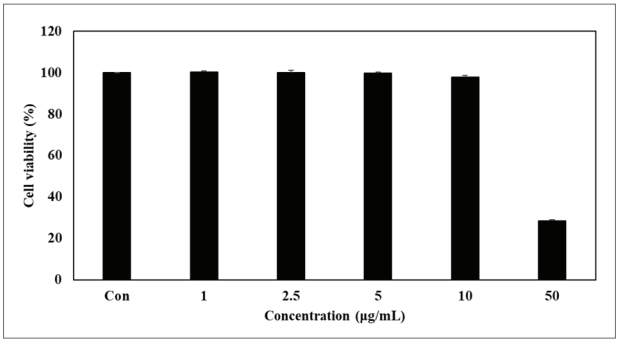

Fig. 4. Inhibitory effects of UPE on IL-6 production in LPS-stimulated RAW 264.7 cells. IL-6 production was determined in RAW 264.7 cells treated with UPE and indomethacin. UPE – ultrasound peanut shell extract. After RAW 264.7 cells were cultured for one day in a six-well plate (3×10^5 cells/well), they were treated with LPS (10 μg/mL) for 2 h. Next, the cells were treated with UPE and indomethacin and measured 18 h later. IL-6 production was measured using an IL-6 ELISA kit. The results are presented as the mean±standard deviation from three measurements (*P < 0.05 vs. the LPS alone treatment group).

NO and PGE_2

To measure the anti-inflammatory effects of UPE in LPS-stimulated RAW 264.7 cells, NO and PGE_2 production was examined using the Griess assay and ELISA, respectively. The results showed that 10 μg/mL of UPE significantly inhibited NO production by 63.17%. Moreover, UPE treatment decreased PGE_2 production (Fig. 3).

IL-6

To determine the levels of proinflammatory cytokines, IL-6 production was investigated in LPS-stimulated RAW 264.7 cells. LPS-stimulated cells caused an up-regulation of IL-6 production, however, the presence of UPE significantly decreased IL-6 levels in a dose-dependent manner (Fig. 4). The inhibitory effect of UPE at a concentration of 10 μg/mL was greater compared with that of indomethacin treatment (64.94±0.77 vs 79.92±0.61).

iNOS and COX-2

Western blot was used to evaluate iNOS and COX-2 production in LPS-stimulated RAW 264.7 cells. The results showed that UPE decreased the expression levels of iNOS and COX-2 in a concentration-dependent manner (Fig. 5). Considering that UPE decreased iNOS and COX-2 production at the protein level, the effect of UPE on iNOS and COX-2 expression at the mRNA level was evaluated in LPS-treated RAW 264.7 cells. The results showed that UPE decreased

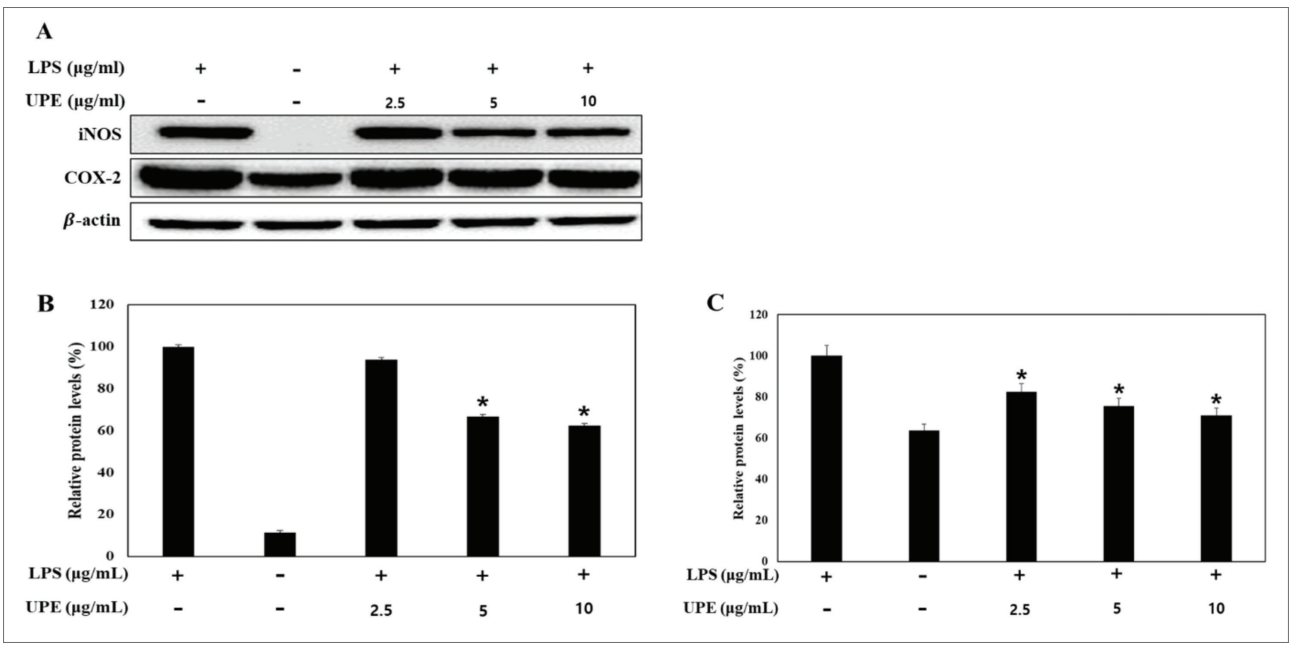

Fig. 5. Effects of UPE on iNOS and COX-2 protein expression levels in RAW 264.7 cells. Representative Western blots (**A**) and relative densitometric quantification of (**B**) iNOS protein expression and (**C**) COX-2 protein expression in RAW 264.7 cells after LPS treatment. After RAW 264.7 cells were cultured (1×10^6 cells/well) for 24 h in a 100 mm culture dish, they were treated with LPS (10 μg/mL) for 2 h. Next, the samples were treated with UPE and measured 18 h later. The results are the mean±standard (*P<0.05 vs. the LPS treatment group).

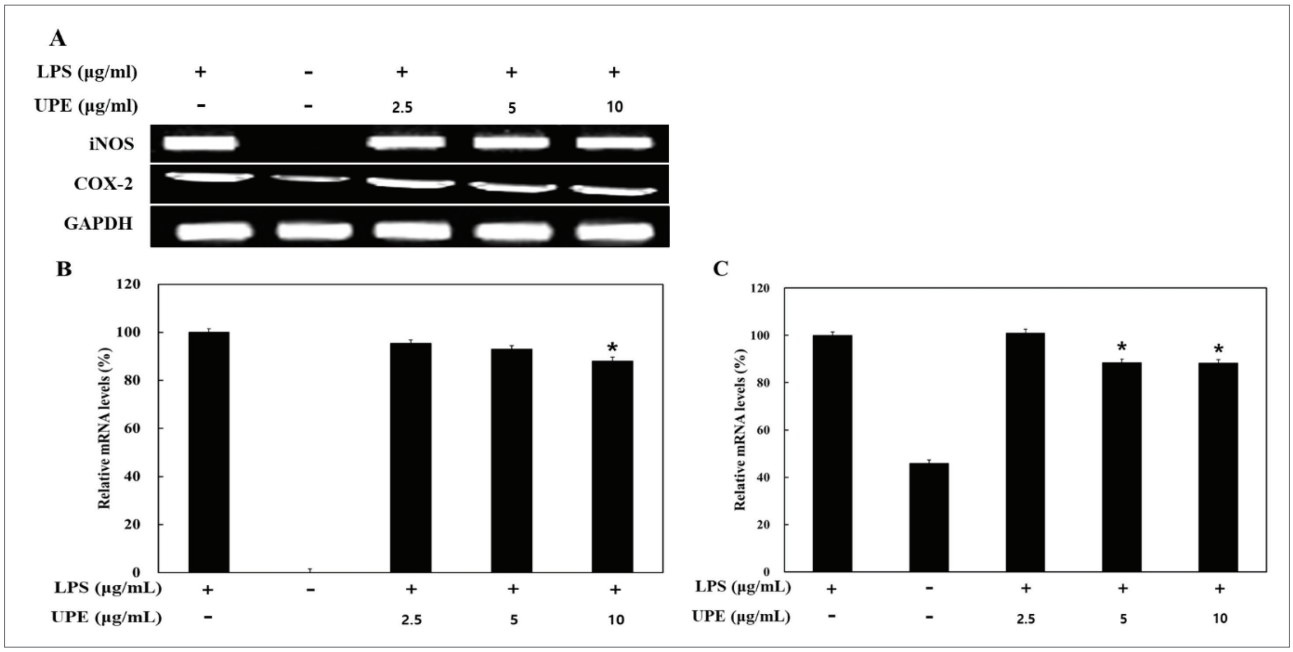

Fig. 6. Effects of UPE on iNOS and COX-2 mRNA expression in RAW 264.7 cells. Representative mRNA expression (**A**) and relative densitometric quantification of iNOS mRNA expression (**B**) and COX-2 mRNA expression (**C**) in RAW 264.7 cells after LPS treatment. After RAW 264.7 cells were cultured (1×10^6 cells/well) for 24 h in a 100 mm culture dish, they were treated with LPS (10 μg/mL) for 2 h. Next, the samples were treated with UPE and measured 18 h later. The results are presented as the mean ± standard deviation (*P<0.05 vs. the LPS alone treatment group).

the expression levels of iNOS and COX-2 compared with the LPS group (Fig. 6). These results confirmed the anti-inflammatory effect of UPE on NO and PEG_2 production induced by iNOS and COX-2 at the protein and mRNA levels.

DISCUSSION

In this study, we focused on the biological effects of peanut shell extract by-products obtained using UAE and demonstrated the antioxidant and anti-inflammatory effects in LPS-stimulated RAW 264.7 cells. Recent studies have explored the potential of peanut shell by-products as sources of bioactive compounds with antioxidant and anti-inflammatory properties. Recycling foods from natural sources is essential for addressing environmental issues related to food waste, which could also enhance food production [25]. Several research groups have investigated plant and food waste to obtain new sources of active agents [26-27]. Peanut by-products have generated increasing interest in developing products with pharmacological activities. We investigated the effects of peanut shell by-products obtained using UAE.

Ultrasound-assisted extraction (UAE) is one of the most utilized technologies for extracting compounds from plant-based foods [28]. UAE has been shown to effectively extract polyphenols from peanut skins, yielding high levels of catechins, procyanidins, and other phenolic compounds [29-30]. These extracts demonstrate significant antioxidant activity and anti-inflammatory effects, including inhibition of COX-2 protein expression and reduction of PGE2 and NO levels [29]. Additionally, peanut skin extracts exhibit anticancer properties in different cell lines [30]. Optimized UAE conditions have been used for extracting compounds with skin-whitening and anti-wrinkle effects, showing promise for cosmetic applications [10]. Furthermore, peanut shells contain valuable amino acids and high levels of luteolin, a bioactive flavonoid [31]. The amount of luteolin extracted from peanut shells using UAE was evaluated to optimize the extraction conditions. In the present study, HPLC demonstrated a 1.8-fold increase in luteolin in UPE compared to other extraction conditions (Supplementary Fig. S1). The luteolin content was approximately 1.96% when 70% ethanol was used as the solvent, whereas the total luteolin content in the extract was 3.57%, measured under identical

extraction duration conditions. Siziya et al. reported that extraction optimization of luteolin from peanut shell extract [32] by ultrasound exposure required lower extraction temperatures and shorter times, resulting in higher flavonoid yields when compared to conventional methods [33-35]. According to Liao et al., ultrasonic power enhanced the solubility of flavonoids with an increment of mass transfer and reduction of solvent viscosity [36]. These findings highlight peanut shell by-products as a source of bioactive compounds for food, pharmaceutical, and cosmetic industries.

Among phytochemicals, plant phenolic compounds are studied because of their antioxidant and anti-inflammatory effects [37]. The antioxidant activity of UPE was analyzed using the DPPH and ABTS assays. UPE exhibited increased DPPH and ABTS radical scavenging activities. According to previous studies, the antioxidant activity of peanut shells is related to luteolin content [38]. The anti-inflammatory effects of peanut shell extract were investigated in LPS-stimulated RAW 264.7 cells. Following treatment with 50 μg/mL UPE, cell viability was assessed. UPE concentrations of 2.5, 5, and 10 μg/mL were non-cytotoxic. LPS stimulation of macrophages causes an increase in NO production by the proinflammatory mediator iNOS [39]. This leads to the inflammatory response and the release of cytokines, including TNF-α and IL-6, which increase PGE_2 production by COX-2 [40]. UPE significantly reduced NO and PGE_2 production in a concentration-dependent manner. Moreover, UPE significantly reduced the expression of iNOS and COX-2 at the protein and mRNA levels in LPS-treated RAW 264.7 cells. The activation of NO and PGE_2 by iNOS and COX-2 during LPS stimulation leads to inflammation [41]. The present study confirmed that peanut shell extraction using ultrasound increased the anti-inflammatory activities associated with the iNOS and COX-2 inhibition. IL-6 is a proinflammatory cytokine that affects homeostatic function by mediating inflammation [42]. UPE inhibited IL-6 expression in LPS-stimulated RAW 264.7 cells, whereas TNF-α was unaffected. These results indicate that UPE reduced IL-6 expression by inhibiting NO and PGE_2 production. In summary, UPE reduced the production of anti-inflammatory mediators, including NO, PGE_2, iNOS, COX-2, and IL-6, in LPS-stimulated RAW 264.7 cells.

Funding: The authors received no specific funding for this work.

Author contributions: This must include a statement of the different responsibilities that specify the contribution of every author. JW Kang: Conceptualization, methodology, investigation, validation, formal analysis, data curation, writing — original draft preparation. IC Lee: Conceptualization, resources, writing — review and editing, supervision, project administration. The authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare no conflicts of interest.

Data availability: Data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kang%20and%20Lee_Raw%20Dataset[1].pdf

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SUPPLEMENTARY MATERIAL

Supplementary Fig. S1. High-performance liquid chromatography showing the changes of luteolin contents in 70% ethanol and peanut shell extract using ultrasound-assisted extraction (UAE).