RESEARCH ARTICLE

Immunomodulatory Effect of *Dioscorea esculenta* L. on *NF-κB*, *TLR-4*, *TNF-α*, and *IL-10* Expressions in LPS-stimulated RAW 264.7 Mouse Macrophages

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Abstract

BACKGROUND: Gene expressions of toll-like receptor 4 *(TLR)-4*, nuclear factor-kappaB *(NF-κB)*, tumor necrosis factor *(TNF)-α*, and interleukin *(IL)-10* are known to have roles in the inflammatory process and affect the regulation of the immune system. A preliminary study showed that *Dioscorea esculenta* L. tuber has immunomodulatory activity against macrophage phagocytosis activity and lymphocyte proliferation. However, the immunomodulatory activity of aqueous extract (AE), polysaccharide fraction (PF), and non-polysaccharide fraction (NPF) of *D. esculenta* L. tubers on these gene expressions have not been elucidated well. Therefore, this study was performed to determine its immunomodulatory activity by utilizing RAW 264.7 cell culture induced by lipopolysaccharide (LPS).

METHODS: RAW 264.7 cells were stimulated with LPS at a concentration of 1 µg/mL for 30 minutes before incubation with non-toxic concentrations of AE, PF, NPF, positive control, and inulin at 25 and 50 µg/mL. *TNF-α*, *IL-10*, *TLR-4*, *NF-κB*, and *β-actin* expressions were evaluated using reverse transcription-polymerase chain reaction (RT-PCR) and were normalized with *β-actin* as an internal control. Triplicate experiments were performed throughout this study.

RESULTS: Treatment with 25 µg/mL NPF significantly decreased the expression of *NF-κB*, *TLR-4*, and *TNF-α* ($p<0.05$). In contrast, treatment of 25 and 50 µg/mL PF significantly decreased the *NF-κB* expression ($p<0.05$). Moreover, only treatment with 50 µg/mL AE exhibited a significant increase in *IL-10* expression ($p<0.05$).

CONCLUSION: Treatment with *D. esculenta* L. tuber stimulated macrophage RAW 264.7 cells via *NF-κB*, *TLR-4*, *TNF-α*, and *IL-10* expressions. NPF at 25 µg/mL has stronger immunomodulatory activity in reducing the expression of genes involved in the inflammatory process that plays a role in regulating the immune system.

KEYWORDS: *Dioscorea esculenta* L., Immunomodulator, IL-10, NF-κB, TLR-4, TNF-α, RAW 264.7 cell

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Introduction

The growing prevalence of diseases makes the body more susceptible to bacterial and viral infections. When the immune system is exposed to substances that are considered foreign, there will be two types of immune responses, namely the innate immune response and the adaptive immune response.(1) Macrophages are important members of the innate immune system and, together with neutrophils, eosinophils, and natural killer (NK) cells, are the first line of defense to identify, eliminate, or attack toxic microorganisms and macromolecules.(2,3) Macrophages are one of the immune cells that can express toll-like receptor

(TLR)-4 receptors that can recognize lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria. It will further signal transduction via TLR-4 and activate the innate immune response, and stimulate various proteins that are important for macrophage function.(4) TLR-4 can stimulate the production of the transcription factor nuclear factor kappaB (NF-κB), which produces various proteins and a number of inflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukins (IL) that play a role in the immune response.(5)

The immune system can be boosted through exercise, adequate rest, and a balanced diet. A particular group of nutrients or foods can provide health benefits by influencing immunological and inflammatory parameters, which are clinically and experimentally proven (referred to as immunonutrients).(6) The immunonutrients in immunomodulators are part of the strategy to modulate the immune response. Immunomodulators are substances that can affect or alter the immune response in the body. (7) Immunonutrients can be sourced from natural products such as tubers, a local food ingredient that some people usually consume instead of rice. Several studies have demonstrated that tubers contain immunonutrients, including dietary fiber, antioxidants, prebiotics, vitamins, and minerals, all of which are known to enhance the health of the digestive tract and support the immune system.(8) Due to their multipharmacological properties, including immunomodulatory and antioxidant effects, natural products and their active metabolites have garnered considerable attention recently as an alternative. Natural products and their active metabolites play a crucial role in treating immunological diseases by modifying immune responses.(9)

Dioscorea esculenta L., or also known as Gembili tubers, are a type of tuber that contains immunonutrients and has been proven to strengthen the immune system. *D. esculenta* tubers' water-soluble polysaccharide (PLA or soluble dietary fiber) content gives them immunostimulant and prebiotic properties. Compared to *Dioscorea alata* tubers, *D. esculenta* tubers have substantially greater quantities of inulin, with values of 23.21% per gram of extract.(10) Glucomannan was found to be 39.49% present in *D. esculenta* tubers in water extract.(11) Non-polysaccharide bioactive substances, such as dioscorine and diosgenin, which can act as immunostimulants, are also present in *D. esculenta* tubers.(12)

In a preliminary study that we conducted, the aqueous extract (AE), polysaccharide fraction (PF), and non-polysaccharide fraction (NPF) of *D. esculenta* L. were found to enhance macrophage phagocytosis activity, with the highest activity observed at 100 µg/mL AE, 100 µg/mL PF, and 12.5 µg/mL NPF. AE, PF, and NPF were also demonstrated to increase lymphocyte proliferation activity, with the most significant enhancement noted at 12.5 µg/mL AE, 12.5 µg/mL PF, and 50 µg/mL NPF.

The previous research was performed on the expression of *D. esculenta* L. genes, especially as an immunomodulator. Therefore, this study was conducted as a follow-up investigation to determine the immunomodulatory activity of the AE, PF, and NPF of *D. esculenta* L. on *TLR-4*, *NF-κB*, *TNF-α*, and *IL-10* gene expressions, which play roles in the inflammatory process and affect the regulation of the immune system. This study was conducted by utilizing RAW 264.7 macrophage cell culture *in vitro*, induced by LPS using the reverse transcription-polymerase chain reaction (RT-PCR) method.

Methods

D. esculenta Extract Preparation

Fresh *D. esculenta* L. tubers were procured from Gundi and Ledokdawan Village, Geyer, Grobogan, Central Java. The tubers were selected for their freshness and maturity, having been harvested 8 to 9 months after planting. *D. esculenta* L. plant was identified and authenticated by a scientific officer at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta. The voucher specimens were deposited in the herbarium (No. 17.12.4/UN1/FFA.2/BF/PT/2023).

The tubers of *D. esculenta* L. were peeled, cleaned, and chopped into small pieces while running water. After that, they were combined with hot water at 80 to 90°C in a 1:4 ratio. Next, the mixture was filtered to obtain the filtrate, which was then cooled. The filtrate obtained is hereinafter referred to as the AE of *D. esculenta* L. A freeze-dryer was used to partially dry this water extract.(13) A 96% ethanol was used to further precipitate the *D. esculenta* L. tubers' aqueous extract. This solution was stored in a freezer at approximately -10°C for 18 hours until a precipitate formed. It was then thawed at 8°C for 2 hours and drawn with a vacuum pump to obtain more precipitate. A freeze-dryer was employed to dry the precipitate, which pertains to the PF of *D. esculenta* L. Meanwhile, a vacuum rotary evaporator concentrated the filtrate for 6–7 hours at 60°C. Subsequently, a freeze dryer was utilized to dry the viscous portion, which is referred to as the NPF of *D. esculenta* L.(14) From the extraction and fractionation of *D. esculenta*

L. tubers, the yields for AE, PF, and NPF were 2.51%, 12.00%, and 2.75%, respectively.

Fourier Transform Infrared Analysis

Freeze-dried AE, PF, and NPF were characterized by their functional groups using Fourier Transform Infrared (FTIR) Model 630 (Agilent Technology, Santa Clara, CA, USA) at a wavenumber of 4000–400 cm^{-1} (without using KBr), with Inulin (Sigma-Aldrich, St. Louis, MO, USA) as a comparison standard.(10) The criteria for identifying "inulin-like" compounds can be observed in the FTIR spectrum, with an important peak in the characteristic inulin band around 935 cm−1.(15)

Cell Culture

The Parasitology Laboratory of the Faculty of Medicine, Universitas Gadjah Mada, provided the RAW 264.7 cells. Incubated at 37°C with a 5% CO_2 supply, the cells were kept in the complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Cat. No. 2646161; Gibco, Waltham, MA, USA), 10% fetal bovine serum (Cat. No. 0001643900; Sigma-Aldrich), and 1% penicillin-streptomycin (Cat. No. 1103915; Gibco).

Cell Viability

The cells were plated at a density of 4.0×10^3 cells per well in 96-well plates, with 100 µL per well, and incubated in a CO_2 incubator at 37°C for 24 hours. The media was discarded, and 100 µL of AE, PF, NPF, a positive control (Imboost, a commercial product containing 250 mg of Echinacea purpurea, PT Soho Global Health, Jakarta, Indonesia), and inulin (Sigma-Aldrich) were added at concentrations of 25, 50, 100, and 300 µg/mL. Next, the media was discarded again, and 100 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Bio Basic, Markham, ON, Canada) was added. After 4 hours, 100 µL of 10% SDS (Merck, Rahway, NJ, USA) in 0.01 N HCl was added as a stopper. Following another 24 hours of incubation, absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 550 nm.(16)

RNA Extraction and cDNA Synthesis

Cells were plated at a density of 1.0×10^6 cells per well in 24-well plates and stimulated with LPS (Sigma-Aldrich) at 1 µg/mL for 30 minutes before incubation with non-toxic concentrations of AE, PF, NPF, positive control, as well as 25 and 50 µg/mL inulin. Total RNA was isolated from the cells using the Total RNA Mini Kit (Geneaid, New Taipei City, Taiwan). A reverse transcription system comprising 2 µL of 5x RT Master MIX (ReverTra Ace® Toyobo, Osaka, Japan), 2 µg of total RNA, and 6 µL of RNase-free water was used to transform the total RNA into cDNA. The reaction was conducted for 15 minutes at 37°C, then incubated for 5 minutes at 50°C, and finally heated for 5 minutes at 98°C.(17) The concentration of the synthesizing cDNA was measured using a NanoDrop instrument (NanoQuant Plate; Tecan Spark, Männedorf, Switzerland).

Gene Expression Analysis

The expression levels of the *TNF-α*, *IL-10*, *TLR-4*, and *NF-κB* were analyzed by taking 2.0 µL of cDNA at a concentration of 100 ng/µL and combining it with 28.0 µL of PCR Master Mix (GoTaq Green; Promega, Madison, WI, USA). This mixture contained 15.0 µL of Master Mix, 1 µL of forward primer, 1 µL of reverse primer, and 11.0 µL of DNase/RNase-free water. The gene expression levels of *TNF-α*, *IL-10*, *TLR-4*, *NF-κB* and *β-actin* were determined using RT-PCR. A thermal cycler (Bio-Rad) was used to apply the following conditions: denaturation at 95°C for one minute; 45 PCR cycles at 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 20 seconds; and final steps at 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. (18) PCR products were analyzed by electrophoresis using 2% agarose and FluoroVue™ (Nucleic Acid Gel Stain; SMOBIO Technology, Hsinchu City, Taiwan) at 10,000X, catalog number NS1000, in a 500 µL vial as a dye. Gels were visualized using densitometry with Gel-Doc (Bio-Rad) software. Band density was evaluated using ImageJ software (NIH, Bethesda, MD, USA). *TNF-α*, *IL-10*, *TLR-4*, and *NF-κB* expression levels were normalized with *β-actin* as an internal control. Primer sequences were based on algorithm-generated sequences from Primer Bank: http://pga.mgh.harvard.edu/primerbank (accessed on 24 September 2024), by entering the target gene ID primerbank on the website by selecting the mouse species. The primers for the *TNF-α*, *IL-10*, *TLR-4*, *NF-κB* and *β-actin* genes are listed in Table 1.

Statistical Analysis

Triplicate experiments were performed throughout this study. Data from all experiments are presented as mean±standard error of the mean (SEM). One-way analysis of variance or the Kruskal–Wallis test was employed to determine the significance between the groups. Statistical analysis was performed using SPSS version 23 (IBM Corporation, Armonk, NY, USA), and $p<0.05$ indicated statistically significant differences.

Table 1. Mouse oligonucleotide primer sequences used in reverse transcriptase-PCR.

Gen	Primer Type	Sequence (5′→ 3′)	Size (BP)
TNF-α	Forward	CCCTCACACTCAGATCATCTTCT	61
	Reverse	GCTACGACGTGGGCTACAG	
IL-10	Forward	GCTCTTACTGACTGGCATGAG	105
	Reverse	CGCAGCTCTAGGAGCATGTG	
TLR-4	Forward	ATGGCATGGCTTACACCACC	129
	Reverse	GAGGCCAATTTTGTCTCCACA	
NF-κB	Forward	ATGGCAGACGATGATCCCTAC	111
	Reverse	TGTTGACAGTGGTATTTCTGGTG	
β-actin	Forward	GGCTGTATTCCCCTCCATCG	154
	Reverse	CCAGTTGGTAACAATGCCATGT	

Results

Effects of AE, PF, and NPF on the Viability of RAW264.7 Cells

The RAW 264.7 cell viability test, when administering AE, PF, and NPF, indicated a reduction in the number of viable cells as the concentration increased. The AE, PF, and NPF groups at concentrations of 25 µg/mL and 50 µg/mL exhibited an average cell viability above 80%. However, in the groups with concentrations of 100 µg/mL and 300 µg/mL, the average cell viability dropped below 80%. In the positive control group, which included a commercial product containing 250 mg of *E. purpurea*, all tested concentrations, namely 25, 50, 100, and 300 µg/mL, showed an average cell viability exceeding 80%. The inulin group consistently maintained an average viability above 80% at 25 µg/mL and 50 µg/mL concentrations (Figure 1).

Effects of AE, PF, and NPF on *TLR-4* Expression in LPS-stimulated RAW 264.7 Cells

The effects of AE, PF, and NPF on *TLR-4* were assessed after LPS treatment and activation. As shown in Figure 2, the expression of TLR-4 in the negative group was higher

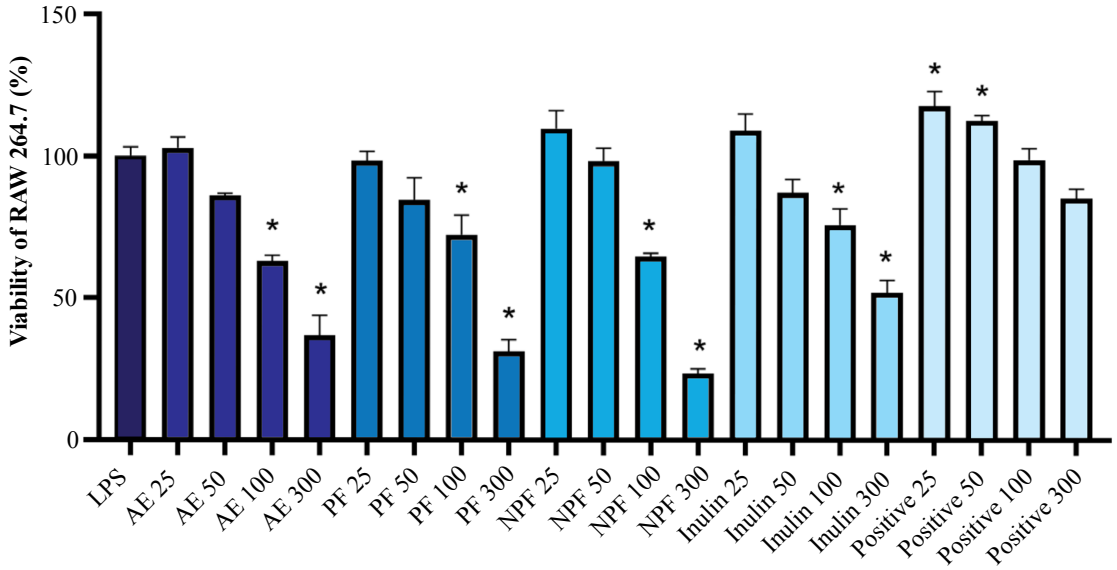

Figure 1. The cellular viability measurements using the MTT assay. The RAW 264.7 cells were incubated with various concentrations of AE, PF, and NPF, along with a positive control for 24 hours, and 1 µg/mL LPS as a negative control. Each histogram represents the mean±SEM from n=3. *Significant differences were compared with LPS (p<0.05).

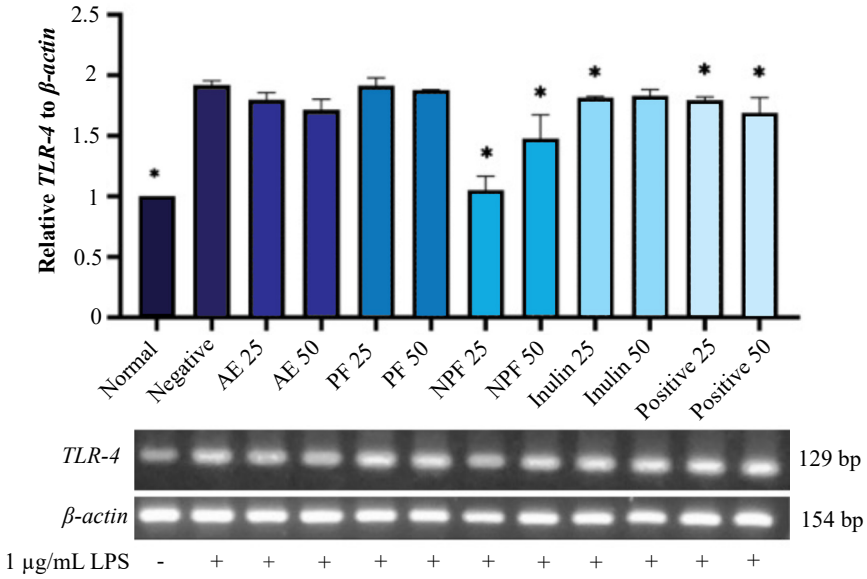

Figure 2. Effects of AE, PF, and NPF on cytokine *TLR-4* mRNA expression in LPS-stimulated RAW 264.7 cells. The values are expressed as the means±SEM of results obtained from at least three independent experiments. *Significant differences were compared with LPS (p<0.05).

than in the normal group (p<0.05). This result indicates that LPS can promote TLR-4 expression. Treatment of NPF at 25 and 50 µg/mL, inulin at 25 µg/mL, and the positive control at 25 and 50 µg/mL significantly decreased TLR-4 expression in LPS-stimulated RAW 264.7 cells (p<0.05).

Effects of AE, PF, and NPF on *NF-κB* Expression in LPS-stimulated RAW 264.7 Cells

The effects of AE, PF, and NPF on *NF-κB* expression were evaluated after LPS treatment and activation. A significant difference was observed when comparing the negative and normal groups (p<0.05). This indicates that the presence of LPS can enhance *NF-κB* expression. As shown in Figure 3, treatment of PF at 25 and 50 µg/mL, NPF at 25 µg/mL, inulin at 25 µg/mL, and the positive group at 25 and 50 µg/mL significantly decreased in *NF-κB* in LPS-stimulated RAW 264.7 cells (p<0.05).

Effects of AE, PF, and NPF on *TNF-α* Expression in LPS-stimulated RAW 264.7 Cells

The effects of AE, PF, and NPF on the pro-inflammatory cytokine *TNF-α* were assessed following LPS treatment and activation. A significant difference was observed when comparing the negative and normal groups (p<0.05). This indicates that the presence of LPS can enhance the

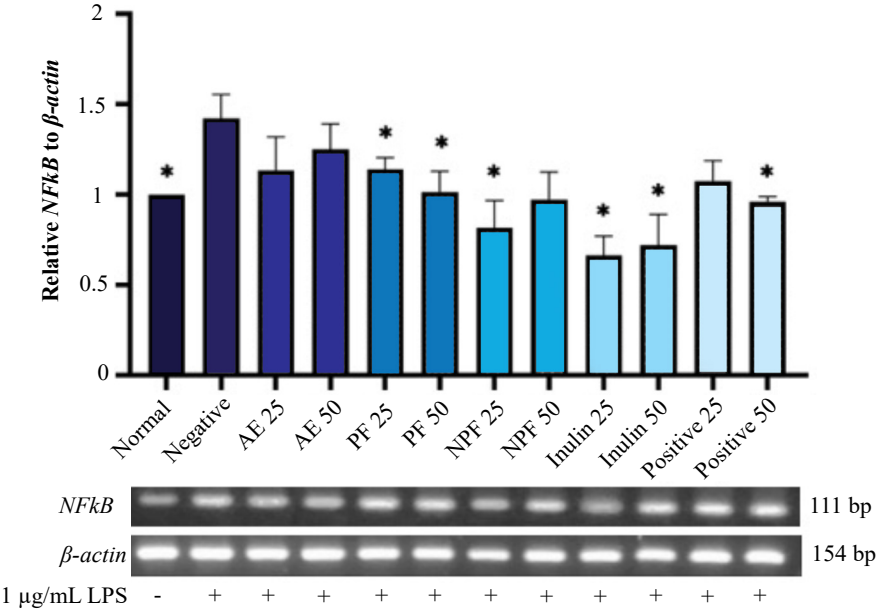

Figure 3. Effects of AE, PF, and NPF on *NF-κB* mRNA expression in LPS-stimulated RAW 264.7 cells. The values are expressed as the means±SEM of results obtained from at least three independent experiments. *Significant differences were compared with LPS (p<0.05).

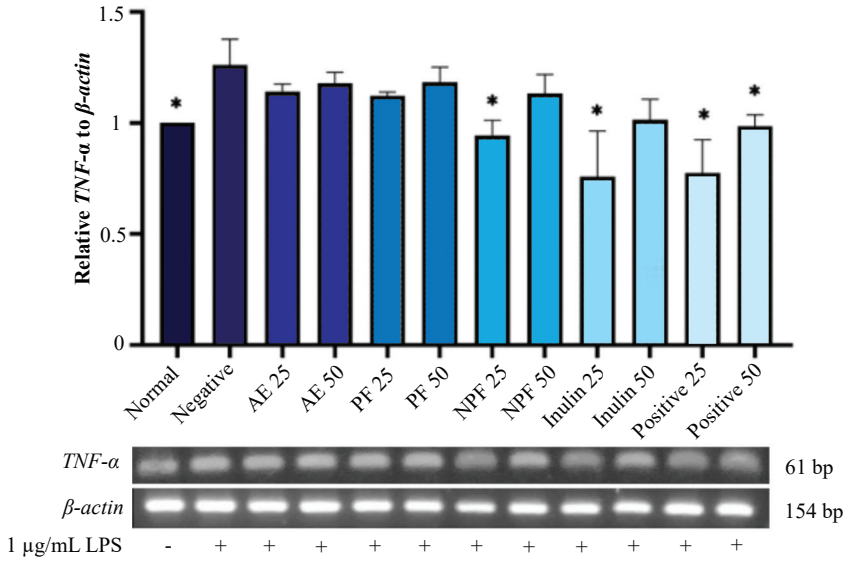

Figure 4. Effects of AE, PF, and NPF on cytokine pro-inflammation *TNF-α* mRNA expression in LPS-stimulated RAW 264.7 cells. The values are expressed as the means±SEM of results obtained from at least three independent experiments. *Significant differences were compared with LPS (p<0.05).

expression of the *TNF-α*. As shown in Figure 4, treatment of NPF at 25 µg/mL, inulin at 25 µg/mL, and the positive group at 25 and 50 µg/mL significantly decreased *TNF-α* in LPS-stimulated RAW 264.7 cells (p<0.05).

Effects of AE, PF, and NPF on *IL-10* Expression in LPS-stimulated RAW 264.7 Cells

The effects of AE, PF, and NPF on the anti-inflammatory cytokine *IL-10* were assessed following LPS treatment and activation. A significant difference was observed between the negative and normal groups (p<0.05). This indicated that the presence of LPS can decrease IL-10 expression. The treatment of AE at 50 µg/mL and positive control at 25 µg/mL significantly increased IL-10 expression in LPS-stimulated RAW 264.7 cells (p<0.05) (Figure 5).

Fourier Transform Infrared Analysis (FT-IR) Results

FT-IR characterization revealed peaks at specific wave numbers identifying inulin, supported by the standard inulin (Figure 6). These characteristic absorption bands include the O-H vibration stretching at a wave number of 3268.88 cm^{-1}, the C-H stretching vibration of the alkane chain at a wave number of 2925.96 cm^{-1}, the C-O-C stretching vibration of the ring at a wave number of 1123.80 cm^{-1}, residual α-D-Glucopyranosyl in the carbohydrate chain at a wave number of 933.70 cm^{-1}, followed by 2-ketofuranose at wave numbers 872.20 and 812.56 cm-1.(10,19,20) The criteria for identifying "inulin-like" compounds can be observed in the FT-IR spectrum, with an important peak in the characteristic inulin band around 935 cm^{-1}. Figure 6 and Table 2 indicated that *D. esculenta* L. PF contained an inulin-like compound,

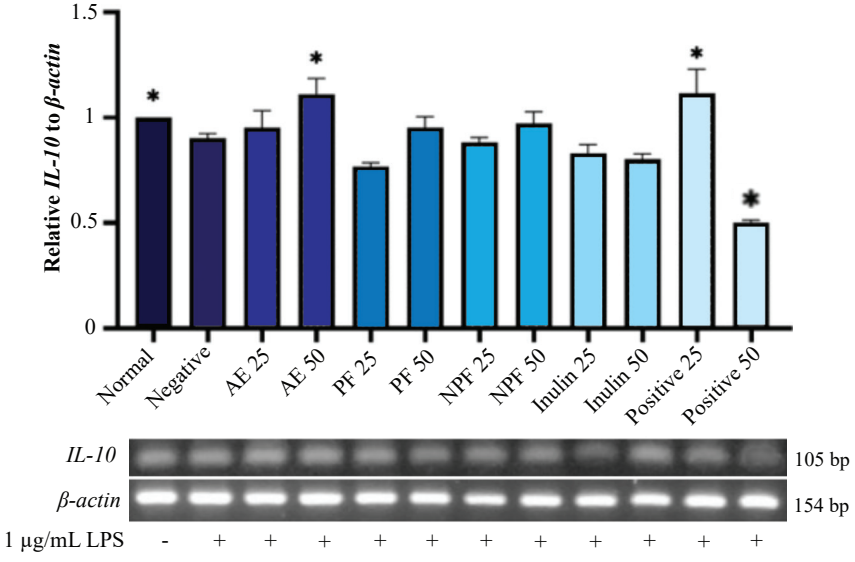

Figure 5. Effects of AE, PF, and NPF on cytokine anti-inflammation *IL-10* mRNA expression in LPS-stimulated RAW 264.7 cells. The values are expressed as the means±SEM of results obtained from at least three independent experiments. *Significant differences were compared with LPS (p<0.05).

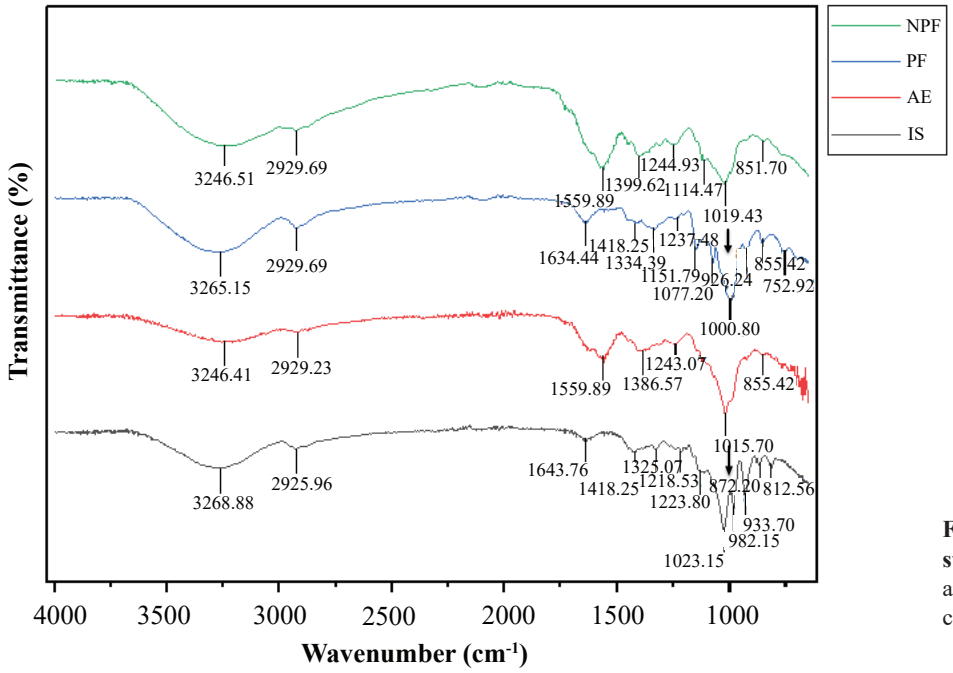

Figure 6. FT-IR spectra of inulin standard, AE, PF, and NPF. Black arrows indicate important peaks in the characteristic inulin band.

based on the similarity of the functional groups with the inulin standard and PF has an important peak in the characteristic inulin band, namely 926 cm^{-1}.

Discussion

The RAW 264.7 cell line is a monocyte-macrophage cell line widely used in immune system research due to its similarity to macrophages produced by bone marrow. These RAW 264.7 cells were derived from mice induced by the Abelson murine leukemia virus.(21,22) In this research, LPS was used to activate RAW 264.7 cells. LPS is found in the outer cell membrane of gram-negative bacteria and can activate macrophages by triggering cellular signaling pathways.(23) LPS induced inflammatory cytokines in RAW 264.7 macrophage cells through the mediation of TLR-4 and NF-κB signaling pathways.(24) Binding of LPS to *TLR-4* induces phosphorylation and ultimately activates *NF-κB*, a factor that regulates DNA transcription to control the expression of genes coding for proteins involved in various biological processes. Cellular stimulation will then induce the transcription of pro-inflammatory genes by producing pro-inflammatory cytokines such as TNF-α. These cytokines can activate nearby non-specific cells, promoting bacterial phagocytosis.(25)

Table 2. Wavenumbers of AE, PF, NPF, and inulin standard.

No.	Wavenumber (cm^{-1})				Functional Groups
	Inulin Standard	AE	PF	NPF	
1	3268.88	3246.41	3265.15	3246.51	O-H
2	2925.96	2922.23	2929.69	2929.69	Asymmetric C-H Stretching vibration
3	1418.25 1325.07 1218.53	-	1418.25 1334.39 1237.48	-	C-O-H
4	1123.80	-	1077.20	1114.47	C-O-C
5	1023.15	1015.70	1000.80	1019.43	C-O
6	933.70	-	926.24	-	Residual α-D-Glcp in the carbohydrate chain
7	872.20 812.56	-	855.42 752.92	-	2-ketofuranose

Innate immune cells utilize both positive and negative feedback mechanisms, some of which are facilitated by cell-to-cell communication, to regulate inflammatory responses. A well-known example is IL-10, a negative regulator that plays a crucial role in reducing and managing inflammation. IL-10 limits the activity of antigen-presenting cells and adaptive immune responses, and it also inhibits the synthesis of pro-inflammatory cytokines and chemokines. The negative regulatory mechanisms of IL-10 are difficult to distinguish due to their complex interactions with other regulatory elements. For instance, the primary paracrine signals that regulate inflammatory responses in macrophages and dendritic cells, including IL-10 generation, are TNF and interferon (IFN)-β, which are activated following TLR-4 stimulation.(26,27) Strict regulation of the inflammatory response is necessary for immune homeostasis. This is how the immune system is modulated.

This study started by cultivating RAW 264.7 cells in media until they reached confluence. Only cell viability between 80% and 95% was utilized for further study. Concentrations that lead to a viability below 80% are considered toxic to the cells.(28) In this study, macrophages remained viable at 80% with AE, PF, and NPF at concentrations of 25 and 50 g/mL, indicating that AE, PF, and NPF at both concentrations are safe for cell survival. However, 100 and 300 µg/mL concentrations of AE, PF, and NPF were considered toxic to LPS-induced RAW 264.7 cells, as their viability dropped below 80%. Subsequently, 25 and 50 µg/mL concentrations of AE, PF, NPF, inulin, and the positive control were chosen for gene expression analysis. Studies on the toxicity test of AE, PF, and NPF of *D. esculenta* L., both *in vitro* and *in vivo*, have not been conducted until now. However, in previous studies, namely the cytotoxic test of ethanol extract, polar fraction, and semi-polar fraction of *D. esculenta* L. on MCF7 cancer cells, it was shown that the higher the concentration of the treated sample (1000; 500; 250; 125; and 62.5 µg / mL), the number of living cells decreased.(29)

The results showed that AE at 25 and 50 µg/mL could not significantly decrease the expression of the pro-inflammatory genes *TLR-4*, *NF-κB*, and *TNF-α*. However, AE at 50 µg/mL can significantly increase the expression of the anti-inflammatory gene *IL-10*. While previous studies on the activity of *D. esculenta* L. tubers concerning *IL-10* gene expression have not been conducted, research has focused on the immunomodulatory effects of *D. alata* L. tubers (one family with *D. esculenta* L. tubers), demonstrating that the methanol extract of these tubers can promote the up-regulation of *IL-10* and *IL-4*, as well as down-regulation of nitric oxide (NO), *IL-2*, interferon *(IFN)-γ*, *TNF-α*, prostaglandin E2 *(PGE2)* levels, and cyclooxygenase *(COX)* activities.(30)

PF at 25 and 50 µg/mL can only significantly decrease the *NF-κB* gene expression. NPF at 25 µg/mL showed a significant decrease in *TLR-4*, *NF-κB*, and *TNF-α*. This is thought to be caused by several metabolite contents that are extracted when the water extract is precipitated using 96% ethanol. Ethanol is a solvent with high solubility (dielectric constant 24), so it is helpful in dissolving all substances, both polar and semipolar.(31) Based on previous research, the semipolar fraction of *D. esculenta* L. contains terpenoids, saponins, alkaloids, and phenolics.(29) In the ethanol extract of *D. esculenta* L., saponin compounds have also been isolated.(32) In the phytochemical preliminary screening that we have conducted, it was shown that the non-polysaccharide fraction of *D. esculenta* L. contains alkaloids and saponins. These compounds are thought to play a role in immunomodulatory activity.

Total saponins from *Dioscorea collettii* have been shown to reduce cytokine production by inhibiting the activation of the TLR4/NF-κB signaling pathway.(33) Alkaloids are also thought to act as immunostimulants by increasing the production of IL-2 and lymphocyte proliferation in culture. Activated lymphocyte cells will activate cytokines such as IL-2 and IFN-γ. These cytokines will activate macrophages to respond to antigens in the body. However, alkaloids can also be cytotoxic, which can cause immunosuppressive activity.(34) This is what is thought to cause an increase in NPF concentration not accompanied by an increase in effect. NPF at 50 µg/mL can only significantly reduce the *TLR-4* gene expression.

In addition, it is suspected that there is an interaction between the metabolites contained so that no effect appears when the concentration is increased. This often occurs in natural materials, because the compound components they contain are not single but consist of various chemical compounds, where these components work together to cause a response. However, with increasing concentration, the number of chemical compounds contained increases, so that there is a possibility of adverse interactions that cause a decrease in the function of the compound content. (35,36) Further research is necessary to better understand the mechanism that makes the relationship between concentration and response non-linear.

In the present study, positive controls used a commercial product containing 250 mg of *E. purpurea*

and inulin. The results showed that *E. purpurea* can cause downregulation of *TLR-4*, *NF-κB*, and *TNF-α*, along with an upregulation of *IL-10*. This result reveals a molecular mechanism in *E. purpurea* that produces anti-inflammatory activity, enabling it to act as an immunomodulator. This conclusion is supported by previous research. *E. purpurea* extract was found to increase the immune response through activation of the NF-κB and MAPK pathways associated with TLR-4, thereby increasing the expression of immunomodulators such as iNOS, COX-2, mPGES-1, IL-2, IL-6, IL-10, IL-1β, IFN-γ, and TNF-α. This suggests that the extract can be considered a potential immunostimulating agent or functional food.(37)

A polysaccharide called fructan, which has a chain length of 2–60 units, is what inulin is. This fiber is categorized as one that dissolves in water but is indigestible by digestive enzymes.(38) This study demonstrated that inulin decreased the expression of the *TLR-4*, *NF-κB*, and *TNF-α* genes but did not increase the expression of the *IL-10*. This result is supported by a previous study, which indicates that inulin influences the immune system in several ways. By inhibiting the NF-κB, ERK 1/2, and JNK pathways, it contributes to IgA secretion. This, in turn, lowers pro-inflammatory molecules (IL-6, IL-12, p40, and TNF-α) by inducing lymphocytes and dendritic cells and boosts immunological responses by activating the complement system.(38) Inulin also stimulates immune cells in Peyer's patches, leading to IL-10 production and activating cytotoxic NK cells.(39)

According to FT-IR analysis, PF shows similarities in functional groups to standard inulin, indicating that PF contains inulin-like compounds. In this study, PF did not demonstrate immunomodulatory activity comparable to standard inulin, specifically in terms of downregulating *TLR-4*, *NF-κB*, and *TNF-α*. This is believed to be because PF does not contain pure inulin, which means it can only provide one of the immunomodulatory activities: the downregulation of *NF-κB* expression. These results can serve as a basis for further studies on inulin isolation and its effectiveness in reducing *TLR-4*, N*F-κB*, and *TNF-α*.

Based on the results of the study, it was shown that the NPF treatment group at 25 µg/mL was the most potent in reducing the expression of *TLR-4*, *NF-κB*, and *TNF-α* genes. This is in line with the TLR-4 and NF-κB signaling pathways, where when these signaling pathways are lowered, the production of TNF-α proinflammatory cytokines also decreases. However, further research is needed to confirm the immunomodulatory activity of EA, PF, and NPF after administration in an *in vivo* model induced by an antigen.

Conclusion

This study provides evidence that AE, PF, and NPF from *D. esculenta* L. tubers demonstrate immunomodulatory activity through different molecular pathway mechanisms. NPF at 25 µg/mL was the most potent in reducing the expression of *TLR-4*, *NF-κB*, and *TNF-α* genes. PF at 25 and 50 µg/mL can lower the expression of the *NF-κB* gene. Additionally, AE at 50 µg/mL increased the expression of the anti-inflammatory *IL-10* gene. These findings indicate that *D. esculenta* L. tubers possess the potential to function as a natural immunomodulator for the development of functional food or nutraceuticals.

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Authors Contribution

IP was involved in plant collection, processing, and experimental work. AN, NF, and MI supervised the overall study. IP is involved in the conceptualization and writing. AN, NF, and MI reviewed and edited the manuscript. All authors read and agreed to the final version of manuscript.

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