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# Optimization of Enzyme-Assisted Extraction from Ginger (*Zingiber officinale*) Leaf and Its Immune-Stimulating Effects on Macrophages

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**ABSTRACT:** The ginger leaves contain terpenoids and phenolic compounds, such as gingerol and shogaol, which exert various physiological effects. This study focused on determining the optimal conditions for an enzyme (Ultimase MFC) extraction to enhance the bioactive components of underutilized ginger leaves using the response surface method. The extracted material was evaluated in terms of its yield and antioxidant capacity (total phenolic content, total flavonoid content, and activities of 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid). As a result, the optimal conditions included an enzyme concentration of 0.1% (v/v), a liquid-solid ratio of 33.939 mL/g, and an extraction time of 4 h. The optimized conditions resulted in an improvement in yield and antioxidant capacity, except for the total phenolic content of ginger leaves, when compared to the reference control extract. Additionally, the possibility of improving immunity was confirmed as nitric oxide and cytokines increased in macrophage cells compared with non-treatment control. Therefore, these extraction conditions enhance the potential industrial value of ginger leaves and underscore their promise as a natural ingredient for functional foods.

**Keywords:** by-product, enzymes, ginger, leaf, macrophages

## INTRODUCTION

The agri-food industry produces substantial amounts of plant byproducts each year during the cultivation and processing of agricultural products. However, many discarded agri-food byproducts and waste materials contain valuable compounds that retain significant functionality and bioactivity. These include various plant parts, such as seeds, leaves, stems, and roots, and represent untapped sources of natural antioxidants (Lourenço et al., 2019). However, at present most byproducts are not exploited as potential sources of beneficial compounds. This is despite the fact that chemical extraction of antioxidant-rich compounds from discarded byproducts has revealed that the inedible parts of fruits and vegetables often possess higher concentrations of bioactive components than the edible parts (Echegaray et al., 2018). Moreover, there is growing interest in discovering new natural materials present in byproducts that may be recovered and used as high-value-added functional materials.

Ginger is a root crop known for its unique aroma and taste, and is widely consumed around the world. The primary active ingredients in ginger include gingerol and shogaol, along with zingiberol, zingiberene, and γ-cardinen (Mao et al., 2019). These active compounds have been found to possess both antioxidant and anticancer properties (Shukla and Singh, 2007). In 2022, 37,000 Mg of ginger were produced, of this ginger leaves accounted for 15,770 Mg, representing 46.2% of the total ginger harvest. Although ginger leaves have been approved for use in foods by the Ministry of Food and Drug Safety in Korea since April 2017, their agricultural utilization rate remains low (Im et al., 2021). Chan et al. (2009) reported that ginger leaves also contain active ingredients similar to those found in ginger roots, including significant quantities of phenols. Moreover, quercetin and kaempferol glycosides have recently been identified in ginger leaves, as well as flavonols (Nam et al., 2021). The potential of ginger leaves for use as a processed food ingredient remains significant, with robust protective effects having been

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demonstrated against various chronic conditions including inflammation, cardiovascular disease, and gastrointestinal disorders (Sasidharan and Menon, 2010).

Considerable research has been performed on the antioxidant activity of plants, including their leaves, and it is known that leaf-associated antioxidants can regulate biological defense and immune functions via similar mechanisms, thereby mitigating the effects of disease and combating aging processes (Kim and Kim, 2005). Although research has been conducted on the physiological activities of ginger leaves (Park et al., 2014), effective methods for extracting and using active ingredients present in ginger leaves remain poorly understood (Chen et al., 2020). In contrast to conventional extraction methods, enzyme-assisted extraction (EAE) can result in faster extraction, lower energy consumption, higher extraction yield, and lower solvent usage (Marić et al., 2018).

In this study, we investigated the ability of different EAE methods to enhance the antioxidant capacity of ginger leaf extracts. In addition, we also used a response surface methodology (RSM) approach to optimize ginger leaf extraction (GLE) using Ultimase MFC then evaluated the immune-stimulating effects of extracts on macrophages.

## MATERIALS AND METHODS

### Materials

Ginger leaves were first harvested from plants grown at the Seasoning Vegetables Research Institute (November 2021). After harvest, we washed harvested leaves three times under running water and discarded plant stems. The leaves were then dried at 60°C for 40 h using a hot air dryer (DS-240BC, Doosung Co., Ltd.) before being ground into a fine powder using a 0.2-mm mesh.

### Chemicals and enzymes

2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), (+)-catechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, Folin-Ciocalteu phenol reagent, gallic acid, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), potassium persulfate ($K_2S_2O_8$), sodium carbonate ($Na_2CO_3$), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich. Ultimase MFC (cellulase), papain, and pectinex (pectinase) were purchased from Novozymes.

### Experimental design for GLE

RSM was applied to determine the optimal extraction conditions for GLE. Related statistical analyses were conducted using Minitab version 17 (Minitab Inc.). Extraction conditions included three independent variables, i.e., enzyme concentration (v/v%), extraction time (h), and the liquid/solid (LS) ratio (mL/g). The values of these variables were established using a Box-Behnken design. The response variables measured included the extraction yield, total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity and ABTS radical scavenging activity. Ultimase MFC, a form of cellulase, was selected due to its antioxidant-related performance after comparing it to papain or pectinase (Supplementary Table 1). GLE was then obtained using Ultimase MFC, with all procedures performed in accordance with the manufacturer's instructions. The optimal temperature and pH for this enzyme were 50°C～60°C and 4.5～6.5, respectively. For all experiments dried ginger leaves were first coarsely ground then finely ground. A mixture of 5 g of finely ground ginger leaf powder, distilled water, and enzyme was kept at 50°C for extraction. The resulting extract was then treated by inactivating the enzyme in boiling water for 30 min, then centrifuging and filtering the resulting mixture using Whatman No. 2 filter paper (Whatman Inc.). To obtain reference control extract (RCE), we used the extraction method used by Chen et al. (2020) with slight modification. Briefly, extractions were conducted at 50°C for 90 minutes using a 1:1:1 ratio of cellulase, papain, and pectinase. The subsequent process was performed in the same manner as for GLE. The yields of all extracts were quantified by weighing both the dried raw material sample and the freeze-dried extract.

### TPC determination

We determined the TPC was determined by modifying the Folin-Ciocalteu method (Dewanto et al., 2002). Briefly, 50 μL of the sample was first mixed with 1 mL of 2% $Na_2CO_3$ at room temperature (RT). Subsequently, 50 μL of 1 N Folin-Ciocalteu reagent was added and the mixture was then left to react at RT for 30 min. After this reaction, 200 μL of each sample was transferred to wells of a 96-well plate, and the absorbance was measured at 750 nm using a microplate reader (SpectraMax M2e, Molecular Devices). The TPC was calculated using gallic acid as a standard and all results are expressed as gallic acid equivalents (mg GAE/g).

### TFC determination

TFC was determined following the method of Zhishen et al. (1999), with modifications. Briefly, 125 μL of sample, 500 μL of distilled water, and 38 μL of 5% $NaNO_2$ were first mixed and left to react at RT for 5 min. Next, 75 μL of 10% $AlCl_3$ was added and the mixture was again left to react at RT for 5 min. Finally, 250 μL of 1 M NaOH was added, and this mixture was left to react at RT for 11 min before the absorbance was measured at 510 nm. TFC was then calculated using catechin as the standard, and all measurements were expressed as catechin equivalents (mg CTE/g).

### DPPH radical scavenging activity

The DPPH radical scavenging activity of extracts was measured as per the protocol described by Blois (1958). Briefly, a 0.2 mM DPPH solution was prepared by diluting DPPH reagent in absolute ethanol, and this sample was then diluted to 5 mg/mL in distilled water. Next, 160 μL of the DPPH solution and 40 μL of sample were mixed and left to react in the dark for 30 min. After reaction the absorbance was measured at 517 nm; the radical scavenging activity (%) was then calculated as the absorbance ratio between groups with and without included sample (i.e., relative to a negative control).

### ABTS radical scavenging activity

We then quantified the ABTS radical scavenging activity as per the method described by Re et al. (1999). Briefly, a mixture of 7.4 mM ABTS solution and 2.6 mM $K_2S_2O_8$ solution was prepared for 16 h in the dark to generate radicals. The ABTS radical solution was diluted with distilled water until it reached an absorbance value at 734 nm of 1.0±0.05. Next, 50 μL of this sample was mixed with 150 μL of the diluted ABTS solution, and the absorbance was measured at 734 nm after incubation at RT for 15 min.

### Cell cultures

RAW264.7 cells, a mouse macrophage cell line, were obtained from the Korean Cell Line Bank and used for all culturing experiments. These cells were first cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin. Cell cultures were then maintained in an incubator (311-TIF, Thermo Fisher Scientific) at 37°C with 5% $CO_2$ and were subcultured.

### Cell viability, nitric oxide (NO) assays, and quantification of cytokine production

Cell viability was assessed using the Ez-Cytox kit (Daeil Lab Services Co.). Briefly, RAW264.7 cells were first seeded onto 96-well plates ($2\times10^4$ cells/well) and were cultured for 24 h. The cells were then treated with GLE and RCE and incubated for an additional 24 h. After this incubation, 10 μL of Ez-Cytox reagent was added to each well, and cells were incubated for a further 3 h. Subsequently the absorbance was measured at 450 nm. Cell viability was expressed as the survival rate of the treatment group relative to a negative control group.

Next, to assess NO production, 100 μL of cell culture supernatant and 100 μL of Griess reagent were combined and allowed to react at RT for 15 min. Absorbance was then measured at 540 nm using a microplate reader.

Finally, the cytokine [tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β] and prostaglandin E2 (PGE2) contents of culture supernatants were measured using an ELISA kit (eBioscience Co.). Here, LPS (1 μg/mL) was used as a positive control to enable activity comparisons.

### Statistical analyses

All statistical analyses were performed using IBM SPSS version 24.0 (IBM Corp.). The means and standard deviation were calculated for each experimental group, and the statistical significance of differences the means of two groups were evaluated using Student's *t*-tests. Moreover, differences among multiple group means were evaluated using one-way ANOVA, followed by Duncan's multiple range test. The statistical significance of differences were evaluated at $P<0.05$.

## RESULTS AND DISCUSSION

### Optimization of GLE conditions

In this study, ginger leaves treated with enzymes were subjected to extraction under 15 conditions designed using RSM. In each case this was followed by the evaluation of antioxidant capacity. Our analysis involved measuring yield and TPC, TFC, DPPH, and ABTS, the results of which are presented in Table 1. Our results show that the yield increased 11.5 times relative to a nonenzymatic control, and ranged from a minimum yield of 3.62%± 0.54% to a maximum extraction yield of 41.66%±2.30%. As expected, the extraction yield increased with higher enzyme concentration and extraction time, and this can be attributed to the decomposition of polysaccharides such as hemicellulose, starch, and pectin within the cell wall (Boulila et al., 2015). In particular, we observed an increase in yield as the LS ratio rose (Jisieike and Betiku, 2020). The tendency for yield to increase with enzyme concentration is consistent with the findings of Nadar et al. (2018). Moreover, according to Chen et al. (2011), cellulase degrades leaf cell walls more effectively than pectinase and significantly transglycosylates flavonol aglycones into soluble polar compounds, which therefore increases extractability. In addition, Ultimase MFC can facilitate the release of bioactive compounds that are loosely bound to cell wall polymers by hydrolyzing (1,4)-β-D-glucosidic linkages that exist between cellulose and other β-D-glucans (Krakowska-Sieprawska et al., 2022). Furthermore, Ultimase MFC shows β-glucosidase activity (Park et al., 2021). Since β-glucosidase has extensive functions, a suitable enzyme can be used to specifically break down the substrate and produce a desired bioactive decomposition product (Singh et al., 2016). Here, we found that TPC increased by 1.4 times and TFC by 1.2 times, and that both TPC and TFC tended to decrease as the enzyme concentration and extraction time increased. These results are consistent with the mass transfer principle and with previous studies that have

**Table 1.** Analytical optimization of the antioxidant capacity and yield of ginger leaf extracts

| Run | Coded | | | Variable | | | Dependent variable | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Enzyme concentration (v/v%) | Extraction time (h) | LS ratio (mL/g) | TPC (mg GAE/g) | TFC (mg CTE/g) | DPPH (%, inhibition) | ABTS (%, inhibition) | Yield (%) |
| 1 | 1 | 0 | 1 | 1.00 | 6 | 10 | 0.85±0.03 | 0.15±0.01 | 50.11±2.67 | 78.41±1.70 | 7.24±1.00 |
| 2 | −1 | 0 | 1 | 0.10 | 6 | 40 | 0.87±0.06 | 0.17±0.01 | 48.50±4.02 | 82.60±3.55 | 25.62±0.47 |
| 3 | −1 | 0 | −1 | 0.10 | 6 | 10 | 0.86±0.10 | 0.16±0.01 | 54.88±1.67 | 82.63±2.29 | 3.62±0.54 |
| 4 | 0 | 1 | 1 | 0.55 | 8 | 40 | 0.78±0.14 | 0.15±0.00 | 39.49±2.09 | 73.30±1.58 | 36.89±0.59 |
| 5 | 0 | 0 | 0 | 0.55 | 6 | 25 | 0.76±0.03 | 0.15±0.01 | 45.40±2.36 | 77.82±2.03 | 25.91±0.28 |
| 6 | 0 | −1 | 1 | 0.55 | 4 | 40 | 0.86±0.08 | 0.16±0.01 | 45.49±3.18 | 78.20±3.16 | 34.07±0.81 |
| 7 | 1 | 1 | 0 | 1.00 | 8 | 25 | 0.76±0.07 | 0.14±0.00 | 43.68±2.55 | 71.76±0.76 | 31.47±0.93 |
| 8 | 0 | 1 | −1 | 0.55 | 8 | 10 | 0.80±0.19 | 0.15±0.02 | 50.23±5.56 | 78.97±6.14 | 6.96±0.60 |
| 9 | −1 | 1 | 0 | 0.10 | 8 | 25 | 0.91±0.08 | 0.17±0.01 | 51.26±3.76 | 82.68±2.93 | 20.35±1.26 |
| 10 | 0 | 0 | 0 | 0.55 | 6 | 25 | 0.80±0.05 | 0.15±0.01 | 42.94±0.88 | 75.41±0.90 | 25.84±0.79 |
| 11 | 1 | 0 | 1 | 1.00 | 6 | 40 | 0.76±0.07 | 0.14±0.01 | 36.65±0.71 | 72.05±5.27 | 41.66±2.30 |
| 12 | 0 | −1 | −1 | 0.55 | 4 | 10 | 0.91±0.12 | 0.17±0.02 | 55.31±4.58 | 83.97±3.19 | 5.01±0.54 |
| 13 | −1 | −1 | 0 | 0.10 | 4 | 25 | 1.03±0.05 | 0.17±0.01 | 55.23±2.03 | 86.05±1.72 | 17.44±0.21 |
| 14 | 1 | −1 | 0 | 1.00 | 4 | 25 | 0.80±0.03 | 0.15±0.01 | 42.92±1.73 | 74.22±1.87 | 28.34±2.22 |
| 15 | 0 | 0 | 0 | 0.55 | 6 | 25 | 0.83±0.09 | 0.16±0.02 | 44.62±1.80 | 77.36±2.32 | 23.92±0.73 |

$X_1$, enzyme concentration; $X_2$, extraction time; $X_3$, LS ratio; LS ratio, liquid-solid ratio; TPC, total phenolic content; GAE, gallic acid equivalent; TFC, total flavonoid content; CTE, catechin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

demonstrated a positive relationship between LS ratios on flavonoid content and extraction yield (Tan et al., 2011; Wong et al., 2013; Elboughdiri, 2018). Next, ABTS radical scavenging activity showed a similar pattern to those of TFC and TPC. Interestingly, DPPH radical scavenging activity was highest (55.31%±4.58%) at intermediate enzyme concentrations and at low LS ratios. Overall, these findings demonstrate the importance of considering multiple factors, including enzyme concentration, extraction time, and LS ratio, to optimize GLE conditions.

Next, we expressed the relationship between the independent variables—i.e., enzyme concentration (v/v%, $X_1$), extraction time (h, $X_2$), and LS ratio (mL/g, $X_3$)—with different response variables as a quadratic polynomial regression equation; these results are expressed in Table 2. TPC was deemed significant with a *P*-value below 0.05; however, it was excluded from the optimal variable set due to its relatively low coefficient of determination ($R^2$=0.611), since equations with $R^2$ values less than 0.7 may have insufficient explanatory power (Kim et al., 2021). Therefore, we designated yield, TFC, ABTS, and DPPH as key optimization variables. Optimization was then carried out to determine which extraction conditions maximized these variables. The corresponding response surface plots for each of these variables are presented in Fig. 1. Optimal extraction conditions were found to be as follows: 0.1% (v/v) enzyme concentration, a 4 h extraction time, and a 33.939 mL/g liquid ratio. The overall satisfaction value was observed to be 0.76. Next, to validate the predicted range of optimization variables and optimized extraction conditions, we then conducted a comparison between the predicted and actual experimental values of the optimization variables (Banik et al., 2020); these results are presented in Table 3. Based on these validation results, the relationships established for the optimization variables concerning yield, TFC, DPPH, and ABTS fell within a 95% confidence interval. This result suggests that the formulated equations for the extraction conditions accurately predict response variables

**Table 2.** Polynomial regression equations for antioxidant capacity and extraction yield of ginger leaf extract

| Response | Polynomial regression equation | $R^2$ | *P*-value |
|---|---|---|---|
| Yield (%) | $Y = -17.37+5.66\ X_1+0.675\ X_2+1.7513\ X_3-5.07\ X_1^2-0.02085\ X_3^2+0.4599\ X_1X_3$ | 0.997 | <0.001 |
| TPC (mg GAE/g) | $Y = 1.0433-0.1384\ X_1-0.02143\ X_2$ | 0.611 | <0.01 |
| TFC (mg CTE/g) | $Y = 0.17366-0.0054\ X_1-0.002444\ X_2+0.000419\ X_3-0.000857\ X_1X_3$ | 0.831 | <0.01 |
| ABTS (%, inhibition) | $Y = 90.49-4.56\ X_1-0.984\ X_2-0.0196\ X_3-0.234\ X_1X_3$ | 0.910 | <0.001 |
| DPPH (%, inhibition) | $Y = 80.95-13.53\ X_1-6.68\ X_2-0.1926\ X_3+9.04\ X_1^2+0.482\ X_2^2-0.262\ X_1X_3$ | 0.947 | <0.001 |

TPC, total phenolic content; GAE, gallic acid equivalent; TFC, total flavonoid content; CTE, catechin equivalent; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; $X_1$, enzyme concentration; $X_2$, extraction time; $X_3$, liquid-solid ratio.

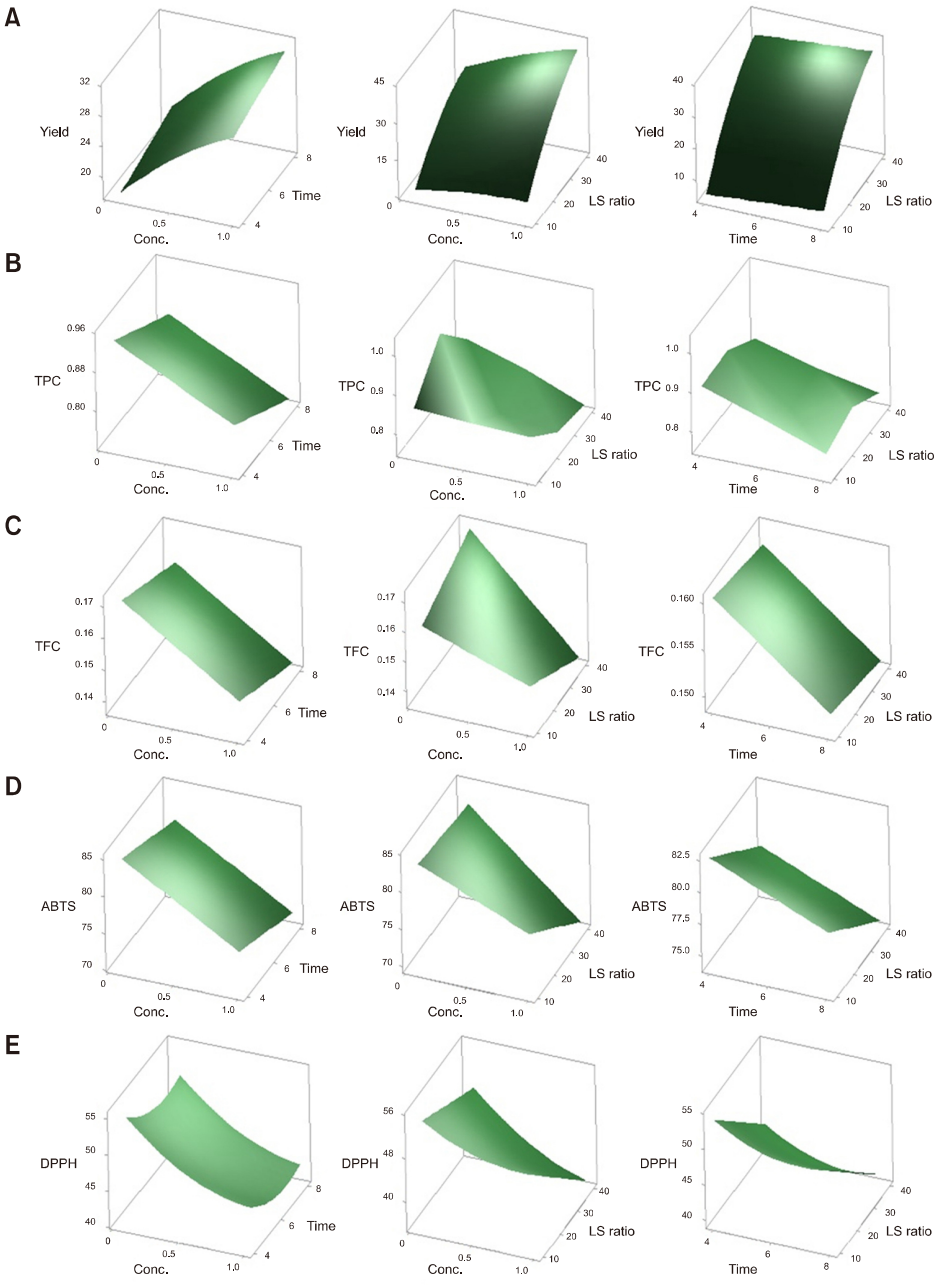


**Fig. 1.** Response surface plots for ginger leaf enzyme extraction methods. (A) Yield (%), (B) total phenolic content (TPC) (mg GAE/g), (C) total flavonoid content (TFC) (mg CTE/g), (D) 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity (%, inhibition), (E) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%, inhibition). Conc., enzyme concentration; LS ratio, liquid/solid ratio (mL/g); GAE, gallic acid equivalent; CTE, catechin equivalent.

**Table 3.** Processing parameters used to optimize the antioxidant capacity and extraction yield of ginger leaf extracts

| Response variable | Optimum extraction condition | | | Predicted value | | Experimental value[1)] |
|---|---|---|---|---|---|---|
| | Enzyme concentration (v/v%) | Extraction time (h) | LS ratio (mL/g) | Fit | 95% CI | |
| TFC (mg CTE/g) | 0.1 | 4 | 33.939 | 0.17 | 0.16~0.19 | 0.17±0.00 |
| DPPH (%, inhibition) | | | | 53.26 | 50.22~56.31 | 51.64±0.81 |
| ABTS (%, inhibition) | | | | 93.69 | 91.32~96.06 | 92.93±0.12 |
| Yield (%) | | | | 22.85 | 20.57~25.12 | 23.54±0.64 |

[1)]Values are presented as mean±SD.
TFC, total flavonoid content; CTE, catechin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; LS ratio, liquid/solid ratio; 95% CI, 95% confidential interval.

(Banik et al., 2019; Deng and Chen, 2019). Thus, the EAE of ginger leaves, which contain high antioxidant capacity and flavonoids, appears to be possible. Next, yield, TPC, TFC, DPPH, and ABTS radical scavenging activity values were compared between GLE and RCE (Fig. 2). Our results showed that these values were significantly

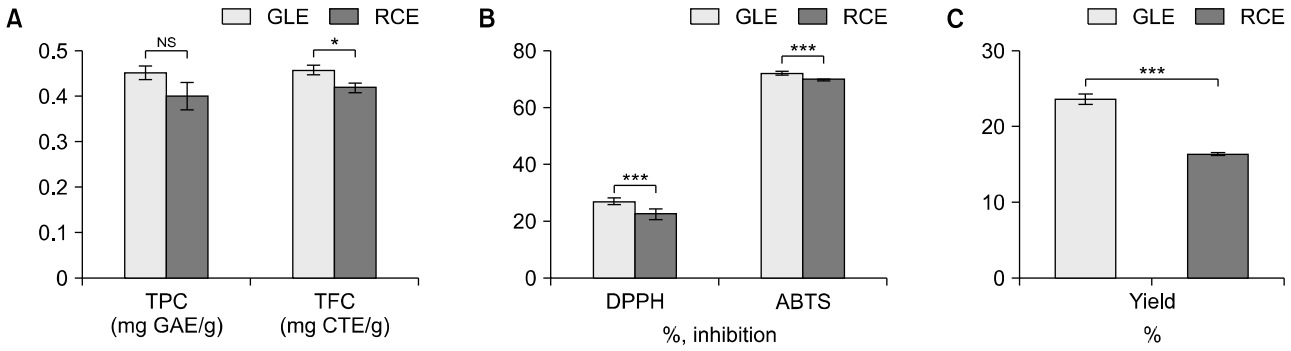


**Fig. 2.** Comparison of antioxidant capacity and yield between ginger leaf extract obtained using the optimized method (ginger leaf extraction, GLE) and the reference control extract (RCE). (A) Total phenolic content (TPC) (mg GAE/g) and total flavonoid content (TFC) (mg CTE/g), (B) 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%, inhibition), (C) yield (%). GLE vs. RCE according to an independent *t*-test (**P*<0.05, ****P*<0.001). GAE, gallic acid equivalent; CTE, catechin equivalent; NS, not significant.

higher for GLE than for RCE ($P<0.05$), except for TPC. Taken together, these results demonstrate that GLE is generally a more effective extraction method than RCE, especially since a higher yield implies the elution of a greater quantity of functional and beneficial components.

### Effect of GLE on immune stimulation in macrophages

To assess the safety of GLE for potential food applications, we then assessed whether the extract exerted an effect on cell viability as measured using RAW 264.7 cells (Fig. 3A). Overall, neither GLE nor RCE exhibited cytotoxic effects within the tested concentration range of 2.5～40 μg/mL. For example, the measured cell survival rate remained ≥95%, reflecting a lack of toxic side effects (Hibbs et al., 1987). Based on this threshold, our data indicate that concentrations of up to 40 μg/mL are safe.

Next, we examined the effect of the extract on macrophage activation. NO is a reactive nitrogen intermediate produced by macrophages in response to stimulation by cytokines or microorganisms, and is therefore generally considered to be a reliable indicator of macrophage activation. In addition, NO is also involved in immune activation and antioxidant activity by minimizing cytotoxicity caused by cell-activating substances and reactive oxygen intermediates (Cho et al., 2010; Byun, 2013). As an active oxygen species, NO is also involved in the regulation of physiological functions including vasodilation, platelet aggregation, and immune response under normal conditions (Hyun, 2011). Furthermore, an increase in NO in macrophages without LPS treatment is considered an immunostimulatory effect (Zhang et al., 2021). In this study, a group in which NO was secreted by treatment with LPS was used as a positive control group; Fig. 3B depicts NO production in response to GLE and RCE treatment in the absence of LPS stimulation. For all samples, we observed an increase in NO production compared to the untreated group. Moreover, GLE increased NO production in a concentration-dependent manner up to 3.6-fold compared to the untreated control, whereas RCE increased NO levels up to 1.6-fold. These results suggest that, in addition to its antioxidant properties, GLE also contributes to immune activation by promoting NO production.

Next, we examined the effect of the extracts on cytokine production. In general, cytokines are proteins secreted by immune cells that regulate various immune cell functions, play roles in effector cell activation, and eliminate external pathogens (Yoo et al., 2014; Park et al., 2017). Increases in cytokine production (within their normal range) generally signify enhanced immune activity, and various natural products have been reported to boost immune activity by increasing cytokine production (Gupta et al., 2022). Among the major cytokines involved in innate immunity, TNF-α, IL-6, and IL-1β are secreted when activated macrophages initiate an immune response. As shown in Fig. 3C～3F, immune-activating cytokines (TNF-α, IL-6, and IL-1β) increased significantly in a concentration-dependent manner in the GLE group. Moreover, both GLE and RCE treatments increased TNF-α and PGE2 levels in a concentration-dependent manner, but TNF-α levels were consistently higher in the GLE treatment. TNF-α contributes to host defense against invading pathogens, and also regulates immune responses by inducing the secretion of other cytokines (Elshal and Hazem, 2022). IL-6 is primarily generated during the initial immune response to infection and/or tissue damage, and the expression of the IL-6 gene is known to be stimulated by other immune factors including TNF-α (Tanaka et al., 2016). In addition, both IL-1β and TNF-α play roles as mediators of the inflammatory response and are significant parts of the cytokine network (Arango Duque and Descoteaux, 2014). In response to GLE treatment, we found that both IL-6 and IL-1β exhibited increases, whereas no such increases were observed for any RCE group. GLE was therefore expected to enhance immune system activation by increasing the expression of TNF-α, IL-6, IL-1β, and PGE2 in macrophages. Our results suggest therefore that GLE stimulates the activa-

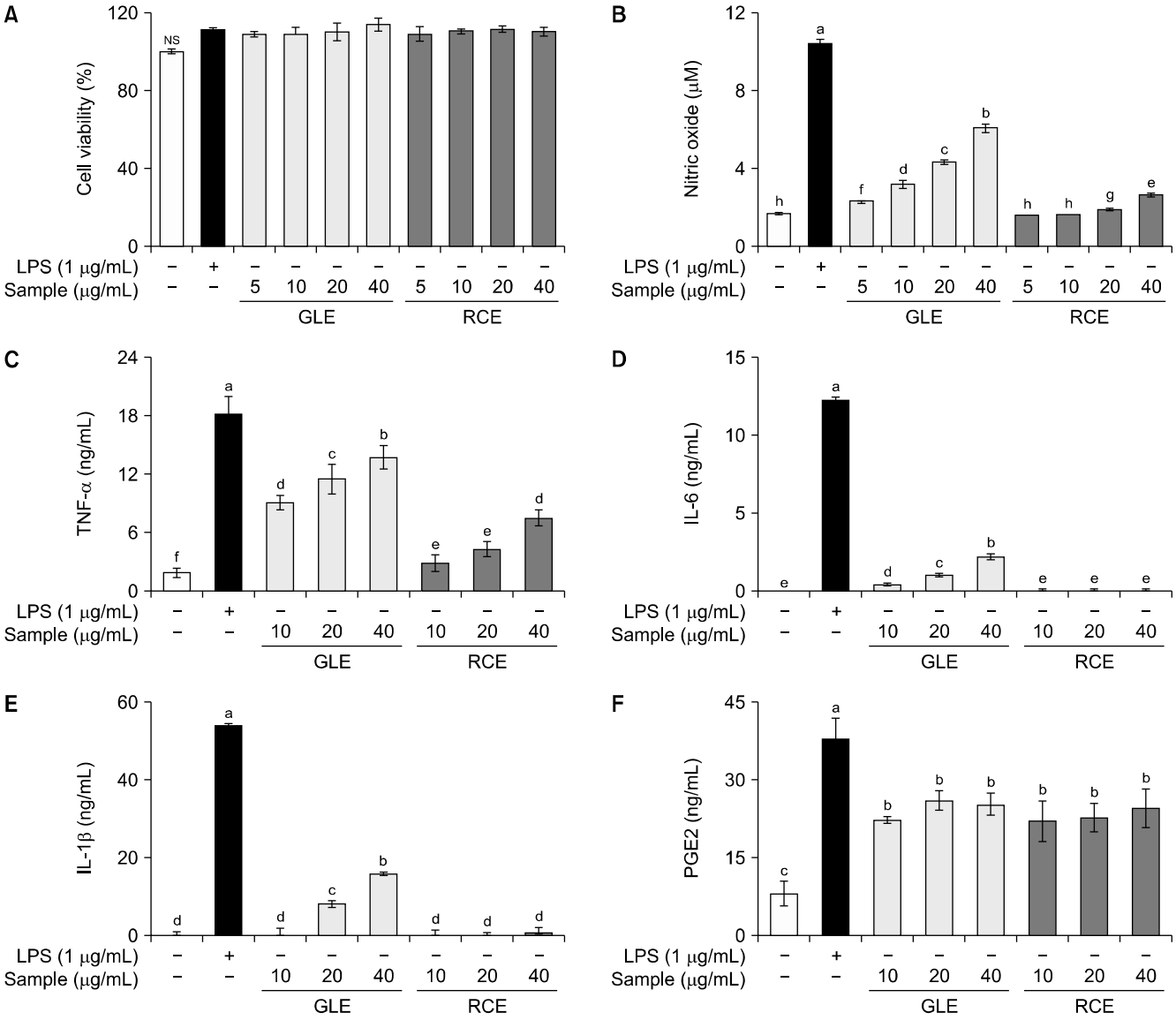


**Fig. 3.** Ginger leaf extraction (GLE) exerts an immunostimulatory effect on macrophages. (A) Cell viability was measured using the Ez-Cytox test. (B) Dose-dependent effects of ginger leaf extract obtained using the optimized method (GLE) and the reference control extract (RCE) on nitric oxide production were determined using a Griess reagent assay. (C) Tumor necrosis factor (TNF)-α, (D) interleukin (IL)-6, (E) IL-1β, (F) prostaglandin E2 (PGE2). Lipopolysaccharide (LPS) (1 μg/mL) was used as a positive control. All results are expressed as mean±SD (n=3). Values with different letters within a column (a-h) differ significantly according to Duncan's multiple range test ($P$<0.05). NS, not significant.

tion of immune cells more effectively than RCE. Taken together, these results also demonstrate the potential for ginger leaves in food industry applications, since a simple functional compound extraction process can yield compounds with valuable and safe medicinal properties. However, further analysis of the physiologically active components present in each extract and the validation of their effects using *in vivo* models are required for improving our understanding of the underlying mechanisms.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Concept and design: JSC, SO. Analysis and interpreta-

tion: HK, MK. Data collection: HK. Writing the article: HK. Critical revision of the article: MK. Final approval of the article: all authors. Statistical analysis: HK, MK. Obtained funding: JSC, YH. Overall responsibility: JSC.

## SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3746/pnf.2024.29.2.228

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