

Synergistic Anti-inflammatory Activity of Ginger Extract and Phlai Oil on Lipopolysaccharide-induced Inflammatory Mediators

Chiramet Auranwiwat, Ph.D.¹, Juwainee Madardam, M.Sc.¹, Titpawan Nakpheng, M.Sc.², Somkamol Intawong, Ph.D.¹, Kanyanat Kaewiad, Ph.D.¹

¹Expert Center of Innovative Herbal Products, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani 12120, Thailand.

²Drug Delivery System Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

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Abstract:

Objective: This study aimed to evaluate the anti-inflammatory activity of the combination of ginger extract and phlai oil on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

Material and Methods: Cell viability, inhibition activity of nitric oxide (NO), tumor necrosis factor- α (TNF- α) and prostaglandin E2 (PGE2) were measured *in vitro* on RAW 264.7 cell cultures stimulated with bacterial lipopolysaccharide.

Results: The IC₅₀ values of ginger extract and phlai oil on RAW 264.7 cells were 1.37 \pm 0.112 mg/ml and 3.79 \pm 0.261 mg/ml, respectively. The production levels of NO, TNF- α and PGE2 was induced by LPS. The inhibition percentages of NO increases in the presence of ginger extract (0.313 mg/ml) and phlai oil (0.625 mg/ml) were 38.22 \pm 0.236% and 25.00 \pm 0.198% in comparison to the control cells. The combination of ginger extract and phlai oil at a ratio 1:1 w/w exhibited statistically significant inhibitory effects on NO production, having an inhibition percentage of 75.98 \pm 0.591%. In the TNF- α assay, an inhibition percentage value of ginger extract (58.74 \pm 0.136%) was higher than that of phlai oil (52.13 \pm 0.238%), while the combination of ginger extract and phlai oil had the highest inhibitory effect (70.03 \pm 0.215%). In the PGE2 assay, the inhibition percentage of the ginger extract-phlai oil combination at a ratio of 1:1 w/w (62.34 \pm 0.187%) was higher than those of both ginger extract (18.21 \pm 0.132%) and phlai oil (34.80 \pm 0.279%).

Conclusion: This study provides scientific evidence in support of the efficacy of the combined use of ginger and phlai oil to reduce inflammatory processes.

Keywords: anti-inflammatory, nitric oxide, prostaglandin E2, RAW 264.7 cell, tumor necrosis factor- α

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Contact: Kanyanat Kaewiad, Ph.D.

Expert Center of Innovative Herbal Products, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani 12120, Thailand.

E-mail: kanyanat@tistr.or.th

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Introduction

The immune system plays a crucial role in recognizing and removing detrimental and alien substances while triggering curative processes via inflammation, an essential component of the body's protective mechanisms. Inflammation can be either acute or chronic. In response to foreign or self-antigens, monocytes extravasate from blood vessels into the injured site and transform into macrophages¹. Phlai oil on lipopolysaccharide (LPS) is an endotoxin that is commonly used in experimental inflammation models. Macrophages play a vital role in the initiation, maintenance, and resolution of inflammation^{2,3}. The activation of macrophages is triggered by proinflammatory mediators like LPS, which then induces the secretion of multiple proinflammatory mediators such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), nitric oxide (NO), prostaglandin E2 (PGE2) and inducible nitric oxide synthase (iNOS), which has been implicated in the development of various inflammatory disorders^{2,4}.

Zingiber officinale Rosc., commonly known as ginger, and *Zingiber cassumunar* Roxb., also known as phlai, are members of the *Zingiberaceae* family. These plants are found in tropical regions. The rhizome of the ginger plant is used as a traditional medicine to treat colds, asthma, and stomachache, and phlai is used externally for the treatment of muscular and joint pain^{5,6}. Literature discusses more than 400 compounds found in the plants of the *Zingiberaceae* family. Some groups of secondary metabolites are produced during these plants' transition from the active growth phase to the stationary phase, especially phenolic compounds and terpenoids⁷. Ginger extract has demonstrated various biological activities such as antioxidant, anticancer, antibacterial, and anti-inflammatory properties⁸. In addition, both ginger and phlai have been found to be important medicinal plants in the reduction of inflammation^{9,10}. Ginger extract and phlai oil possess a diverse array of bioactive compounds, including gingerols, zingerone, shogaols, and sesquiterpenes, which contribute to their medicinal

properties. Furthermore, these compounds may exhibit synergistic effects when combined with other plants in the *Zingiberaceae* family. In both Chinese and Japanese medicine, fresh ginger is prized for its warming properties and its effectiveness in treating coughs and nausea, while dried ginger is recommended for digestive system disorders^{11,12}. In addition, extracts from the ginger rhizome have long been employed in the Malay traditional culture to address a wide range of conditions, including inflammatory and pain-related illnesses, worm infestations, and diarrhea. The rhizomes of *Zingiber cassumunar* Roxb., commonly referred to as "cassumunar ginger", are extensively utilized across tropical Asia for their carminative and stomach-stimulating properties, as well as their effectiveness against diarrhea and colic. In Laos, the phlai rhizome is used to treat abscesses, fever, colic, diarrhea, and other intestinal disorders, and as a depurative and poison antidote, while in Thailand, compounds made from phlai rhizomes are taken to manage asthma and used as the main ingredient in massage oil to relieve muscle pain^{13,14}.

In this study, the anti-inflammatory effects of ginger extract and phlai oil were investigated on RAW 264.7 cells stimulated with LPS. The impact of ginger extract and phlai oil on the expression of the inflammatory mediators NO, TNF- α , and PGE2 was assessed in RAW 264.7 cells.

Material and Methods

Materials

RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC[®], CRL-2278) (USA). Chemicals for the determination of nitric oxide production and toxicity were purchased from Invitrogen[™], USA. TNF- α (Abcam, Cambridge, UK) and PGE2 (Cayman Chemicals, Ann Arbor, USA) were measured using commercial ELISA kits according to the manufacturer's instructions.

Extraction

Fresh ginger rhizomes were washed, sliced, and dried in an oven at 50°C. The dried ginger was blended and

kept in a sealed plastic bag at room temperature. The ginger powder was extracted using ethanol and the percolation method. The solvent was filtrated and concentrated under reduced pressure to obtain a crude ginger extract.

Fresh phlai rhizomes were washed and sliced into small pieces. The phlai was extracted using steam distillation for 6 hours. The distillate was partitioned with dichloromethane and the residue water removed using sodium sulfate (Na_2SO_4). The solvent in the extract was removed with a rotary evaporator to provide a light-yellow oil.

High Performance Liquid Chromatography (HPLC) determination of 6-gingerol and 6-shogaol in ginger extract

The method for the determination of 6-gingerol and 6-shogaol was modified from the one presented by Kajsongkram et al. 2016¹⁵. The mobile phase involved the mixture of acetonitrile and deionized water, and the detection happened at the level of 282 nm.

Gas chromatography mass spectrometry for the determination of terpinene-4-ol in phlai oil

The active substance in phlai oil was analyzed using the gas chromatography-mass spectrometry technique. The separation of compounds was performed on a Mega-5MS (30m x 0.25 mm, 0.25 μm) machine. The oven temperature was set at 60°C for 2 minutes and then increased to 240°C at a rate of 3°C/minute, while the injector temperature was 180°C. Then the mass spectrometer was set at 230°C, and the split ratio was 1:20.

Preparation of extract samples

The compounds to be tested were initially dissolved in dimethylsulfoxide (DMSO), and then Dulbecco's Modified Eagle Medium (DMEM) was added to make solutions in a series of concentrations with a dilution factor of 2. The final concentrations of ginger extract that the cells received were 0.040, 0.080, 0.160, 0.310, 0.630, 1.250, 2.500, and 5.000

mg/ml. For phlai oil, the final concentrations were 0.078, 0.156, 0.313, 0.625, 1.250, 2.500, 5.000, and 10.000 mg/ml. The ginger extract-phlai oil combination was prepared by mixing concentrations (>80% cell viability) of ginger extract and phlai oil at a ratio of 1:1 w/w.

Cell cultures

The RAW 264.7 cells were cultured in 75 cm² culture flasks using DMEM supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin. The cells were incubated at 37°C, 5% CO₂, and approximately 95% relative humidity. The cells were grown the day before the experiment. The cells with 75–80% confluence were removed from the flasks via trypsinization (0.25% v/v trypsin-EDTA).

Cell viability assays

Cell viability was determined using a methyl-thiazolyl-tetrazolium (MTT) assay. Cells were plated at a density of 2×10^5 cells/well in 96-well cell culture plates. One hundred microliters of MTT (10 mg/ml) was added to each well, and the wells were incubated at 37 °C for 2 h. Then 100 μl DMSO was added to each well to dissolve the formazan. After that, the cells were incubated at 37 °C for 10 min and absorbance was read at 570 nm. There was a cell viability of at least 80% in the tested samples taken for study. The calculation for % cell viability followed the equation: % cell viability = [OD sample / OD control] × 100.

Nitric oxide assays

The NO production in the RAW 264.7 cells was measured by its stable metabolite nitrite using the Griess reaction. The LPS from *Escherichia coli* 0111:B4 purified with trichloroacetic acid extraction (Sigma, Australia) was used to stimulate NO production. Briefly, RAW 264.7 cells (density 2×10^5 cells/well) were seeded on a 96-well cell culture plate (Corning Costar, Sigma, Australia) and incubated for 24 h, which was followed by pre-treatments with individual

and combined extracts in 0.1% v/v DMSO. After incubation for 2 h, LPS (10 µg/ml) was added to the cells and co-incubated for another 18 h. After stimulation, 100 µl of the cell supernatant was collected and mixed with the Griess reagent (1% v/v sulfanilamide in 5% v/v phosphoric acid and 0.1% v/v N-1-naphthylethylenediamine dihydrochloride in Milli-Q water) for NO measurements at 540 nm using a microplate reader.

TNF-α assays

The secretion of TNF-α from the RAW 264.7 cells was estimated using ELISA kits according to the manufacturer's protocol. Briefly, 2×10^5 cells were cultured and activated with LPS. Sample supernatants were obtained at the indicated time points and subjected to ELISA. Color development was measured at 450 nm, and concentrations were expressed as pg/ml.

PGE2 assays

RAW 264.7 macrophages were seeded in a DMEM phenol red-free medium in 96-well plates at a density of 2×10^5 cells/well for 24 h. Then the medium was replaced with fresh DMEM containing either 10 µg/ml of LPS alone or LPS with various concentrations of the test compounds. The cells were further incubated for 24 h. The PGE2 production was determined using a PGE2 EIA monoclonal colorimetric assay kit following the manufacturer's instructions.

Statistical analysis

All data are expressed as mean±S.D. ($n \geq 3$). The differences between groups were analyzed using one-way ANOVA. Values of p -value<0.05 were considered statistically significant.

Results

Effects of ginger extract, phlai oil, and the ginger extract-phlai oil combination on cell viability

The study of the ethanolic extract of ginger using percolation provided an extraction yield of 13.54% w/w.

The levels of 6-gingerol and 6-shogaol were measured using HPLC, and yielded the results of 11.28% and 0.82%, respectively. The steam distillation of phlai oil provided a yield of 1.23% w/w. The gas chromatography mass spectrometry finding of terpinene-4-ol in phlai oil was 8.66%.

The cytotoxicity of ginger extract and phlai oil were tested on RAW 264.7 cells using MTT assays, based on an inhibitory concentration at 50% growth (IC_{50}). The IC_{50} values of ginger extract and phlai oil were 1.37 ± 0.112 mg/ml and 3.79 ± 0.261 mg/ml, respectively. Cells with 80% cell viability were selected for the combination experiment. The results showed no significant difference in cell viability (>80%) between treatment with ginger extract (0.313 mg/ml) and phlai oil (0.625 mg/ml) (Table 1, Figure 1). However, the combination of ginger extract (0.313 mg/ml) and phlai oil (0.625 mg/ml) in the ratio of 1:1 w/w resulted in high cell viability ($85.15 \pm 0.86\%$). Based on this finding, these concentrations were selected for further study.

Table 1 Cell viability percentages of ginger extract (mg/ml) and phlai oil (mg/ml) on RAW 264.7 cells

Sample	Concentration	Cell viability (%)
SDS (%)	0.1	35.520 ± 0.625
Ginger extract (mg/ml)	0.039	118.236 ± 1.233
	0.078	105.198 ± 2.946
	0.156	100.197 ± 2.934
	0.313	91.023 ± 1.499
	0.625	78.151 ± 2.048
	1.250	63.421 ± 0.861
	2.500	28.502 ± 2.316
	5.000	14.663 ± 2.817
IC_{50} (mg/ml)	1.37 ± 0.112	
Phlai oil (mg/ml)	0.078	110.674 ± 2.351
	0.156	107.334 ± 3.753
	0.313	104.430 ± 3.324
	0.625	96.626 ± 3.921
	1.250	72.151 ± 2.852
	2.500	63.421 ± 3.578
	5.000	38.502 ± 0.614
	10.000	34.663 ± 1.236
IC_{50} (mg/ml)	3.79 ± 0.261	

SDS=sodium dodecyl sulfate, IC =inhibitory concentration

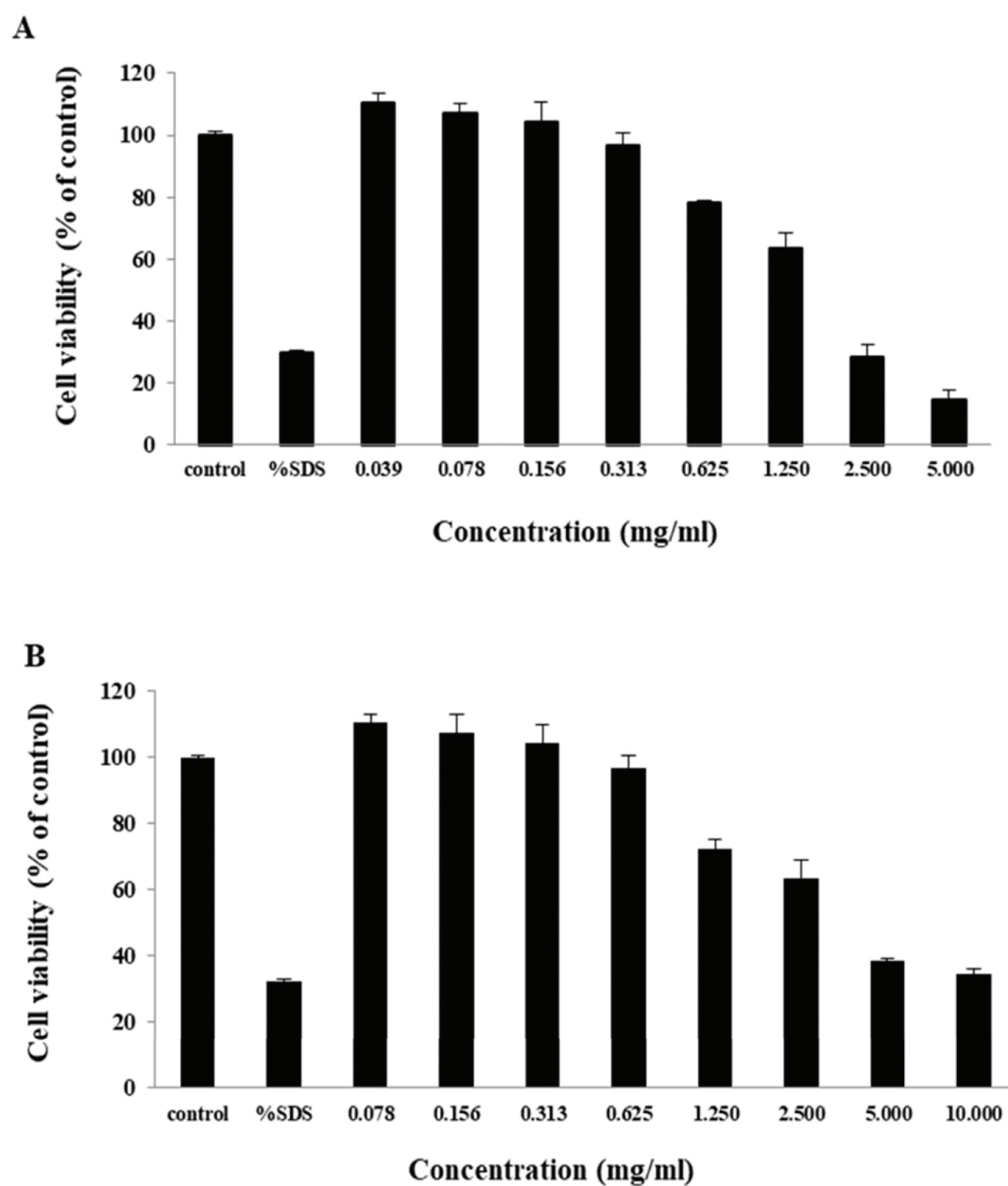


Figure 1 Effects of ginger extract (A) and phlai oil (B) on the viability of RAW 264.7 cells. After culturing the cells with ginger extract (0.039–5.000 mg/ml) or phlai oil (0.078–10.000 mg/ml), cell viability was measured using MTT assays after 24 h.

Ginger extract, phlai oil, and the ginger extract-phlai oil combination inhibited the production of inflammation-related cytokines

When LPS was administered to RAW 264.7 macrophages, the NO, TNF- α , and PGE2 production increased dramatically. The effect of ginger extract, phlai oil, and the ginger extract-phlai oil combination treatments on NO, TNF- α , and PGE2 production in LPS-stimulated cells was investigated, and results are presented in Table 2 and Figure 2. As shown in Figure 2A, the stimulation of RAW 264.7 cells with LPS for 24 h lead to a dramatic increase in NO production from the basal level of 2.22 ± 0.010 nmol/ml to 24.82 ± 0.011 nmol/ml. The percentages of NO inhibition at 0.313 mg/ml of ginger extract and 0.625 mg/ml of phlai oil were calculated at $38.22 \pm 0.236\%$ (15.34 ± 0.023 nmol/ml) and $25.00 \pm 0.198\%$ (18.62 ± 0.159 nmol/ml), respectively. The percentage of NO inhibition yielded by the combination of ginger extract and phlai oil at the ratio of 1:1 w/w was $75.98 \pm 0.591\%$ (5.96 ± 0.021 nmol/ml), indicating a strong synergistic effect between the two compounds.

Figure 2B summarizes the TNF- α production on RAW 264.7 cells after being treated with ginger extract, phlai oil, and the ginger extract-phlai oil combination. The combination sample significantly stimulated TNF- α production in the RAW 264.7 cells. The stimulation of RAW

264.7 cells with LPS increased the secretion of TNF- α to the level of 368.73 ± 0.070 pg/ml. The treatment of RAW 264.7 cells with the ginger extract-phlai oil combination showed a $70.03 \pm 0.215\%$ (110.49 ± 0.040 pg/ml) inhibition level of TNF- α activity. The percentages of TNF- α inhibition with ginger extract and phlai oil were $58.74 \pm 0.136\%$ (152.11 ± 0.02 pg/ml) and $52.13 \pm 0.238\%$ (176.50 ± 0.042 pg/ml), respectively.

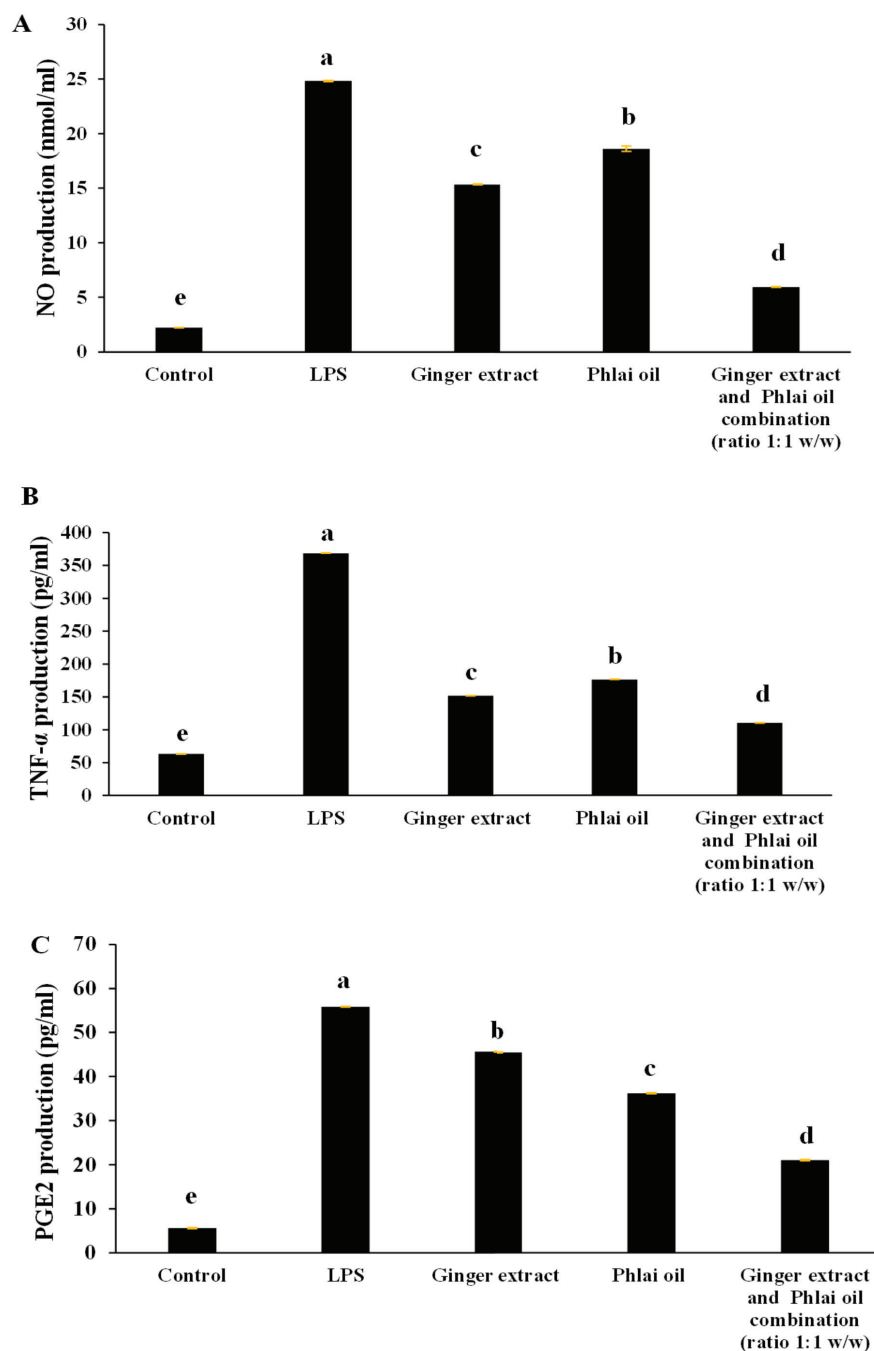
To analyze the effects of ginger extract, phlai oil, and the ginger extract-phlai oil combination on LPS-induced PGE2 levels in RAW 264.7 macrophages, the cells were treated with ginger extract, phlai oil, and the ginger extract-phlai oil combination and then incubated with LPS. Figure 2C shows that the PGE2 levels were significantly inhibited by the treatment with the combination of ginger extract and phlai oil in the ratio of 1:1 w/w as compared to each individual extract. The LPS treatment of RAW 264.7 cells induced the production of PGE2 up to a level of 55.82 ± 0.003 pg/ml, while the unstimulated cells produced a level of only 5.62 ± 0.001 pg/ml. The treatment of cells with the combination sample resulted in a percentage of PGE2 inhibition of $62.34 \pm 0.187\%$ (21.02 ± 0.001 pg/ml), while the ginger extract treatment showed an inhibition percentage of $18.21 \pm 0.132\%$ (45.65 ± 0.020 pg/ml) and that of phlai oil an inhibition percentage of $34.80 \pm 0.274\%$ (36.39 ± 0.051 pg/ml).

Table 2 NO, TNF- α , and PGE2 production according to treatment on LPS-stimulated macrophages

Treatment	Concentration	NO production (nmol/ml)	TNF- α production (pg/ml)	PGE2 production (pg/ml)
Negative control	–	2.22 ± 0.010^e	63.42 ± 0.011^e	5.62 ± 0.001^e
LPS (mg/ml)	0.01	24.82 ± 0.011^a	368.73 ± 0.070^a	55.82 ± 0.003^a
Ginger extract (mg/ml)	0.313	15.34 ± 0.023^c	152.11 ± 0.020^c	45.65 ± 0.020^b
Phlai oil (mg/ml)	0.625	18.62 ± 0.159^b	176.5 ± 0.042^b	36.39 ± 0.051^c
Ginger extract-phlai oil	1:1	5.96 ± 0.021^d	110.49 ± 0.040^d	21.02 ± 0.001^d

*Mean ratio 1:1 of mixed sample between ginger extract (0.313 mg/ml) and phlai oil (0.625 mg/ml)

^{a-e}Means within the same column with different letters indicate a significant difference (p-value \leq 0.05)



LPS=lipopolysaccharide, NO=nitric oxide, TNF- α =tumor necrosis factor- α , PGE2=prostaglandin E2

Figure 2 Effects of individual extracts and their combination on cytokine production in LPS-stimulated RAW 264.7 cells. The RAW 264.7 cells were treated with 0.313 mg/ml ginger extract, 0.625 mg/ml phlai oil, and the combination of ginger extract and phlai oil in the presence or absence of LPS (10 μ g/ml) for 24 h. The production of NO (A), TNF- α (B), and PGE2 (C) was measured in the culture supernatants. The data with different letters indicate significant differences (p -value<0.05) between treatments.

Discussion

There is a growing demand for the use of herbal remedies as natural agents in the treatment of various inflammatory diseases. Several phytochemicals found in herbs, such as flavonoids, alkaloids, lactones, polysaccharides, glycosides, and diterpenoids, have been identified for their potential anti-inflammatory properties. NO is a free radical that plays a crucial role in both cell death and survival, and it exerts various pro-inflammatory effects¹⁶. The expression of iNOS in macrophages is induced by specific triggers and contributes to the pathophysiology of inflammatory diseases¹⁷. TNF- α is a potent pro-inflammatory cytokine that plays a significant role in inflammation as well as in cell differentiation, proliferation, and apoptosis¹⁸. Meanwhile, prostaglandins (PGs) are important prostanoids that play a crucial role in a wide range of physiological and pathological processes¹⁹. The expression of cyclooxygenase-2 (COX-2), which leads to the production of PGE2 that, in turn, primarily regulates inflammatory and immunological events, is significantly increased in response to inflammatory stimuli such as bacterial LPS endotoxin, cytokines, and carcinogens.

Ginger and phlai oil have extensive medicinal histories. They are used in traditional medicine as carminatives, antipyretics, and in the treatment of pain, rheumatism, and bronchitis, offering numerous health benefits¹⁹⁻²². These extracts include their anti-inflammatory properties, immune-boosting effects, and pain-relieving abilities. Both ginger and phlai oil have undergone extensive preclinical and clinical research, exploring their individual anti-inflammatory properties²²⁻²⁵. However, little is known regarding their activity when combined with each other in regulating inflammation, which is commonly seen as their application in traditional medicine. To our knowledge, these research findings provide the first evidence that specific compositions of ginger and phlai oil exhibit a synergistic inhibition of proinflammatory mediators induced by LPS in

RAW 264.7 cells. Synergy is defined as a combined effect of two agents that is larger than the sum of the effects of the individual agents²⁶. In pharmacology, synergistic combinations can lead to improved efficacy, reduced toxicity, and the enablement of a multi-target mode of action²⁶.

The results of this study showed that both ginger and phlai oil extracts dissolved in DMSO were able to inhibit LPS-induced NO production as well as the production of TNF- α and PGE2 in murine macrophages. Previous studies have reported the anti-inflammatory properties of ginger extracts²⁷⁻²⁹, although their results vary depending on the specific cell type and inflammatory stimuli used. Hong and Oh and Shimoda et al. reported that ginger extracts or their constituents affect the nitric oxide production by LPS-activated cells in a dose-dependent way in RAW 264.7 cells^{27,28}. In respect to ginger extract, it has been observed that 6-shogaol exhibits a stronger inhibition of PGE2 production compared to 6-, 8-, and 10-gingerols in RAW 264.7 cells stimulated with LPS³⁰. Gingerols and ginger analogues have been suggested to have distinct mechanisms of action in inhibiting PGE2. Specifically, one study reported that gingerols inhibit the induction of the COX-2 gene³¹. Concerning the phlai extract, treatment with *Z. cassumunar* Roxb. extract has been found to decrease NO secretion significantly³². A major part of the pale amber-colored oil obtained from *Z. cassumunar* Roxb. consists of monoterpenes with terpinen-4-ol as the main constituent³³. In this study, the determination of terpinene-4-ol content in phlai oil using gas chromatography mass spectrometry found a total terpinene-4-ol amount of 8.66%.

The decrease of NO levels after treatment with *Z. cassumunar* Roxb. extract could be the result of the antioxidant properties of the extract. Aupaphong et al. reported that phlai reduces inflammation in human dental pulp cells by lowering the COX-2 and PGE2 production³⁴. It is possible that phlai constituents might decrease the production of inflammatory mediators, including the

prostaglandin E_2 of dental pulp cells via interfering with NF- κ B activation. Kim et al. reported that two analogs isolated from *Z. cassumunar* Roxb. exhibited very strong inhibitory activity against LPS-stimulated NO production and TNF- α release in RAW 264.7 cells³⁵.

Ginger and phlai are known for their anti-inflammatory, antioxidant, analgesic, and digestive properties. They are commonly used to alleviate nausea, indigestion, inflammation, and pain. Other plants in the *Zingiberaceae* family, such as turmeric (*Curcuma longa*) and galangal (*Alpinia galanga*), also possess similar therapeutic properties. Combining extracts from these plants may provide a broader range of bioactive compounds and potentially enhance the overall efficacy of the ensuing herbal formulations. Moreover, the ginger and turmeric combination in the specific ratio of 5:2 (w/w) has been found to exhibit synergistic activity against LPS-induced proinflammatory pathways. Shogaols (specifically 6-s, 8-s, and 10-s) and curcumin have also been identified as key compounds responsible for reducing the expression of major proinflammatory mediators and cytokines³⁶. Similar finding have also been reported for a popular herbal formula, *Salvia miltiorrhiza* Bge. (Danshen) and *Panax notoginseng* Burk. (Sanqi), where a mixture containing the two principal active compounds from each herb did not exert any significant activity, whereas potent synergistic effects were observed for the mixed extracts in a cell model of angiogenesis³⁷.

Conclusion

The extracts of ginger and phlai oil combined at the ratio of 1:1 w/w synergistically reduced the LPS-induced NO, TNF, and PGE2 production in RAW 264.7 cells. Interestingly, this combination also displayed a higher inhibitory activity against proinflammatory mediators and cytokines, which may contribute to anti-inflammatory activity. In summary, this study provides evidence at production levels to support the combined use of ginger extract and phlai oil with a

synergistic approach to reduce proinflammatory mediators, the mechanism of which was found in this study to be at least partially related to interactions among the key bioactive compounds.

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Conflict of interest

The authors report no conflicts of interest in this work.

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