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Review Article

RAW 264.7 Macrophage Cell Line: *In Vitro* Model for the Evaluation of the Immunomodulatory Activity of Zingiberaceae

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ABSTRACT

The immune response plays an essential role in the body's defense against infection. Macrophages are promising targets against which to screen agents that modulate immune responses. *In vitro* analysis, primarily using cell lines, is preferred to screen bioactivity initially. RAW 264.7 cells comprise a model macrophage cell line close to primary murine macrophages. Indonesian people often use the Zingiberaceae family or "empon-empon" in the favorable treatment of several diseases, including immune disorders. This review highlights several studies of Zingiberaceae using RAW 264.7 cells, focusing on the observed immunomodulatory activity and assay methods. The research on RAW 264.7 macrophage cell line in Zingiberaceae was gathered using Scopus, PubMed, ScienceDirect, and Google Scholar from 2012-2021. Based on the reviews, the Zingiberaceae family has immunomodulatory effects by increasing or decreasing inflammatory mediators such as NO, IL-6, TNF-α, IL-1β, IL-10, and phagocytosis activity in RAW 264.7 cells. Several methods can assess the activation of RAW 264.7 cells, including cell viability assays using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT); measuring nitric oxide production (Griess reaction), cytokine production (enzyme-linked immunosorbent assay [ELISA]), and phagocytosis (neutral red uptake assay); and detecting DNA (real-time quantitative polymerase chain reaction [qPCR]). The information provided in this review presents avenues for future investigations of the immunomodulatory activity of Zingiberaceae.

***Keywords*:** immunomodulatory activity, Zingiberaceae, *in vitro*, macrophages, RAW 264.7 cells

Introduction

The human body is frequently exposed to foreign molecules such as pathogens, toxins, and pollutants that can affect health and homeostasis. The immune response is a vital defense mechanism against infections. The innate immune response is induced immediately following infection and does not include immunological memory. Numerous cells are involved in the innate immune response, such as phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils, natural killer (NK), and innate lymphoid cells.[1] Monocyte-derived macrophages are crucial for defense against infections as they process and present antigens, kill pathogens, remove debris, and secrete mediators of inflammation.[2,3] As components of the primary immune response to antigens, macrophages are essential for innate and adaptive immunity, tissue remodeling, wound healing, and tissue homeostasis. The activation of macrophages has a crucial function in host survival and tissue repair by mediating inflammation and cytokine production.[1] RAW 264.7 cells are frequently used as a macrophage model for the *in vitro* evaluation of immunomodulatory activity.[4]

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Immunomodulatory agents can potentially treat immunological dysfunctions such as hypersensitivity, autoimmunity, infections, and cancers. Immunomodulators consist of immunostimulants and immunosuppressants, whose regulatory functions include raising immunity and suppressing an overactive immune system, respectively.[5] In addition, immunomodulators may significantly improve quality of life. Natural products have been used as alternative therapeutic agents to reduce the side effects of drug resistance. Recently, herbal medicines were reported to have various pharmacological and therapeutic effects, including immunomodulatory effects, when used directly or through extraction processes. Herbal medicine, which has a role as an immunomodulatory activity, has been used to activate immunity against pathogens.[6,7] Indonesians use members of the Zingiberaceae family, called "empon-empon," and various plants to maintain wellness by stimulating the immune response. This review summarizes the current methods using RAW 264.7 macrophage cells to evaluate Zingiberaceae's immunomodulatory activity.

Methods

The literature search was performed using Scopus, PubMed, ScienceDirect, and Google Scholar to search for articles published in the English language. The following keywords were used "RAW 264.7 cells" AND "*in vitro*" AND "Zingiberaceae" or "each name of plants such as *Alpinia galanga* (L.) Willd.". The inclusion criteria in this review were original articles from 2012-2021 and used Zingiberaceae family research. These articles are only *in vitro* experiments using RAW 264.7 cells. Articles were excluded from primary articles, which are conference articles and the thesis. *In silico* and *in vivo* experiments were excluded as a reference. Only the full text of relevant articles was included after screening titles and abstracts.

Results and Discussions

RAW 264.7 cell line as a macrophage model

RAW 264.7 cell lines are derived from tumors induced in male BALB/c mice by transforming functional macrophage cell lines with Abelson murine leukemia virus.[8,9] Macrophage models derived from mice (*Mus musculus*) are preferred because of the low variability result parameter experiments between mice and humans.[10] A study found that RAW 264.7 cells were close to primary murine macrophages, possibly affecting macrophage maturation. Therefore, RAW 264.7 cells are commonly used to evaluate the immune system. Additionally, these cells are commercially available, easy to culture, and can be expanded for large-scale screening.[4]

As antigen-presenting cells (APCs) of the innate immune system, macrophages can display antigens bound by major histocompatibility complex (MHC) molecules. In addition, they can capture allergens in specialized endosomes/lysosomes and process them into linear peptides expressed on MHC molecules.[11] Macrophages are classified into the M1 and M2 subgroups. M1 macrophages are pro-inflammatory cells that produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) upon activation. On the other hand, M2 macrophages produce anti-inflammatory cytokines such as interleukin (IL)-4 or IL-10.[12]

Comparison of RAW 264.7 cells and other macrophage cell lines

Macrophages are crucial in eliminating mycobacteria and are utilized as models for studies of mycobacterial host-pathogen interaction.[10] Several macrophage models have been proposed to study the immunomodulatory effects of various compounds.[12] These macrophage models include both primary macrophages, including human monocyte-derived macrophages (hMDMs) and mouse bone marrow-derived macrophages (BMDMs), and cell lines, including THP-1 and U937 among human cell lines and RAW 264.7 and J774 among murine cell lines.[10] A study found that the levels of phagocytosis of a *Leishmania* strain in THP-1 cells were slightly higher than in RAW 264.7 and J774 cells. However, THP-1 cells require much manipulation, and their high replication rates complicate their maintenance and lead to practical difficulties in staining images. RAW 264.7 cells are more robust than THP-1 cells, require less manipulation, and are less sensitive to stress during cultivation. Thus, in the absence of a mouse model, THP-1 cells form an excellent alternative macrophage model.[13]

RAW 264.7 culture conditions

As immortal cells, RAW 264.7 can proliferate indefinitely compared with primary cells. Furthermore, the culture environment is a crucial factor influencing RAW 264.7 cell survival, including conditions such as the culture medium used, CO_2 and O_2 levels in the incubator, and the number of times a cell culture has been subcultured.[4,12]

The passage of RAW 264.7 may genetically drift over time, causing different inflammatory responses.[4] Although immortalized cell lines are used via passaging, passaging effects can enhance the probability of phenotypic and genotypic changes.[14] Cells at high passage numbers differ significantly from those at lower passage numbers in terms of morphology, growth, and differentiation ability.[15] Although macrophages are uniquely found in all tissues and organs, their phenotype varies depending on their physiological state.[16] Interestingly, macrophage cells show plasticity and can polarize and differentiate into osteoclasts, Kupffer cells, or dendritic cells. The American Type Culture Collection (ATCC), the leading global supplier of cell lines, recommends using RAW 264.7 for up to 18 passages because a study reported that RAW 264.7 can differentiate into osteoclasts until passage no. 18. Therefore, confirmation of RAW 264.7 stability across passages is crucial for accurate data collection and interpretation.[17] High plasticity as characteristic of macrophages is functional analysis to evaluate macrophage processes. This study concluded that RAW 264.7 cells were stable until passage no. 30, with the crucial stage being between passage no. 15 and 20. Thus, RAW 264.7 cells should not be used after passage no. 30. Therefore, high plasticity may influence data interpretation of macrophage activation, including phagocytosis and nitrogen oxide production.[12]

Fetal calf serum (FCS) 10% as the supplement in the culture medium of RAW 264.7 cells was affected by the inflammatory response, including the stability, uptake, and metabolism of putative immunomodulators. The RAW 264.7 cells were incubated with 21% O_2. Different types of macrophage cells can be essential factors for culture conditions because they have various proliferative capacities. The immunomodulators' incubation time may also affect their cells' correlation. In this assay, the immunomodulators were incubated for 7 h with whole human blood and peripheral blood mononuclear cells (PBMC) but 24 h with RAW 264.7 cells.[4]

Induction of macrophage activation

Based on pathogen-specific immune responses, RAW 264.7 cells showed similar immune gene expression against Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus* or *Streptococcus uberis*) bacteria. However, the epitope of the Gram-positive pathogen may not be recognized by the transmembrane domain of the toll-like receptor (TLR). *E. coli*-challenged murine RAW 264.7 cell lines increased TNF mRNA concentrations. *E. coli* strongly activates nuclear factor kappa B (NF-κB) in various cell types such as RAW 264.7, epithelial cells (pbMEC and MAC-T), and mammary-derived fibroblasts (pbMFC). Therefore, *E. coli* was recommended for the activation of macrophages.[18]

Lipopolysaccharide (LPS), a pathogenic endotoxin, is recognized by TLRs, mainly TLR4, and induces several signaling pathways to activate macrophages.[19] In addition, LPS consists of a lipid A moiety and polysaccharides of the inner and outer cores. LPS is widely used as an inducer in *in vitro* models of inflammation.[20] LPS-induced macrophages lead to NF-κB activation, phosphorylation of mitogen-activated protein kinases (MAPKs), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway induction.[21] In addition, activation of macrophages is associated with the immunostimulatory phenotype. Activated macrophages produce inflammatory molecules such as TNF-α, IL-6, and especially nitric oxide (NO) that are important in immune regulation, cellular differentiation, and host defense. The IL-2 and IFN-γ cytokines also directly relate to the phagocytosis of macrophages.[22]

Pyroptosis is defined as cell lysis that occurs upon immune cell activation to defend against pathogenic invasion. Glycolytic inhibitors can suppress pyroptosis via the glycolysis pathway. LPS-induced RAW 264.7 cells cultured in a high-glucose medium (4.5 g/l) triggered pyroptosis in longevity. LPS concentration also affected apoptosis to pyroptosis in cell death. A concentration of 10 ng/mL LPS for 72 h induced phenotypic polarization into oxygen-independent glycolysis and pyroptosis. At about 10 ng/mL of LPS, extracellular acidosis was decreased. Furthermore, pro-inflammatory cytokines were released when activated macrophages were associated with metabolic polarization to glycolysis, and the increased extracellular pH was related to the glycolytic rate.[23]

Signaling pathways in macrophage activation

TLRs are among the largest classes of pattern recognition receptors (PPRs), especially in innate cells.[24] TLR4 is the primary receptor in RAW 264.7 cells. The major adaptor protein of TLR4 is myeloid differentiation primary response gene 88 (MyD88), which leads to downstream signaling.[25] The hydrophobic pocket of myeloid differentiation protein 2 (MD2) directly binds to five lipid chains of LPS associated with TLR4. The homodimer of the TLR4-MD2 complex on the cell surface then recruits central adaptor proteins such as MyD88, which is linked to IL-1 receptor-associated kinase (IRAK)4.[26] IRAK4 phosphorylates IRAK1 at Thr-209/Thr-387. The degradation of IRAK1 in the plasma membrane promotes transforming growth factor-β-activated kinase 1 (TAK1) activation in the cytosol, which mediates the activation of MAPK and NF-κB pathways.[27]

TLR4 activation triggers a signaling cascade that promotes several downstream steps such as NF-κB nuclear translocation, activator protein-1 (AP-1) activation, MAPK signaling, phosphatidylinositide 3-kinase (PI3K)-Akt,[28] and nuclear factor (erythroid-derived 2)-like 2 (Nrf2).[29] NF-κB regulates the expression of some immune genes that encode cytokines like TNF-α, IFN-γ, IL-6, and IL-1β. Activation of NF-κB occurs through the enhancement of the phosphorylation of inhibitor kappa B alpha (IκBα) and the NF-κB p65 and p50 subunits.[28] MAPKs,

serine/threonine-specific protein kinases, play a crucial role in innate immune responses and the production of inflammatory mediators, including iNOS, IL-1β, and TNF-α in macrophages. The phosphorylation levels of MAPK signaling, such as extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, are evaluated by Western blot analysis. The phosphorylation of ERK1/2, JNK, and p38 promotes the expression of inflammatory mediators.[2]

AP-1 is a transcription factor that regulates gene expression in an inflammatory immune response. The activation of AP-1 includes the homogeneous or heterogeneous dimerization of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, Fra-1, Fra-2, and FosB).[28] Nrf2 modulates immunity by inducing a cellular oxidative stress response[30] and regulating inflammatory signaling.[29] The JAK/STAT signaling pathway also plays an essential role in the pathogenesis of autoimmune disorders such as rheumatoid arthritis (RA). Several cytokines can indirectly increase the activation of the JAK/STAT signaling pathway by regulating TLR4 and NF-κB signaling.[25] However, LPS does not directly promote STAT activation.

Activation application of RAW 264.7 in Zingiberaceae

The focus on traditional medicines has been increasing over the past few decades. Plants are the most used traditional medicines in human history. The World Health Organization (WHO) declares that 80% of the populations of Asian and African countries still widely use herbal medicine as first-line treatment.[31] Additionally, herbal medicines are safe and efficacious as per individual testimonials.[32]

In this regard, Indonesia is rich, with more than 1,000 plants growing in the distribution area. Local people have used traditional medicine to treat many diseases throughout history, including immune diseases.[33] Zingiberaceae is a famous plant family in the tropical region and a component of the traditional medicines used for several diseases.[6] A study reports that compounds derived from Zingiberaceae show anti-inflammatory activity.[34] "Empon-empon" is a group of rhizomes that consists of various members such as turmeric ("kunyit"), ginger ("jahe"), aromatic ginger ("kencur"), and Javanese ginger ("temulawak"). "Empon-empon" is still very popular in Indonesia as the community believes across generations that it is effective as a herbal medicine for treating various diseases, including as an immunomodulator.

Several studies regarding the modulation effect and assay of RAW 264.7 cells of Zingiberaceae are present in Table 1. *Alpinia galanga* (L). hydroalcoholic extracts and *Amomum compactum* Sol. ex Maton ethanol extracts have immunomodulatory effects of increasing NO, TNF-α, IL-6, and COX-2 production.[25,35] This study showed that *Boesenbergia rotunda* (L.) Mansf, *Curcuma aeruginosa* Roxb., *Curcuma zanthorrhiza* Roxb., and *Zingiber officinale* var. Rubra plays a role in reducing NO production in LPS-induced RAW 264.7 cells.[36–39] NO is a crucial endogenous molecule for immune responses and pathogen killing that is involved in both innate and adaptive immune responses.[40]

Research on the immunomodulatory activity of *Curcuma longa* L. (*C. longa*) using RAW 264.7 cells has been the most widely studied. Based on the study results, *C. longa* plays a role as an immunostimulant and immunosuppressant by increasing and decreasing immune assay parameters. Aqueous extracts and polysaccharide fractions of *C. longa* increase NO production in LPS-induced RAW 264.7 cells.[41–43] In addition, propylene glycol extracts of *C. longa* increase phagocytosis, IL-1β, TNF-α, IL-10 production in pathogen-induced RAW 264.7 cells.[44,45] Turmeronol A, turmeronol B, and n-butanol fraction of the aerial part of *C. longa* reduce inflammatory mediators via the NF-κB signaling pathway.[46,47]

Curcuma mangga Val. & Zijp ethanol extracts have immunosuppressive activity by reducing the expression of the *il-6*, *il-1β,* and *tnf-α* genes in LPS-induced RAW 264.7 cells.[48] Research by Lee *et al*.[49] states that curcuzedoalide in methanol extract of *Curcuma zedoaria* (Christm.) Roscoe (*C. zedoaria*) reduces NO and COX-2 production in LPS-induced RAW 264.7 cells. Guaiane sesquiterpene lactone in *C. zedoaria* chloroform extract decreases *iNOS*, *cox-2*, and *tnf-α* expression in LPS-induced RAW 264.7 cells.[50]

The content of diarylheptanoid in *Kaempferia galanga* reduces NO production in LPS-induced RAW 264.7 cells.[51] In addition, *Kaempferia galanga* contains isopimarane diterpenoid compounds, which can reduce the *tnf-α* and *cox-2* expression and NO production.[52]

Ginger has two varieties: white ginger (*Zingiber officinale* Roscoe) and red ginger (*Zingiber officinale* Rubra). The content of linalool, borneol, zingiberene, and zingerone was significantly different in red ginger than in white ginger.[53] Saanin *et al*.[54] and Arunporn Itharat.[55], evaluated the immunomodulatory effect of *Zingiber officinale* Roscoe ethanol extracts by enzyme-linked immunosorbent assay (for cytokines) and Griess reaction (for NO production). Ethanol extracts of *Z. officinale* Roscoe decrease NO, TNF-α, IL-1β, IL-6, and COX-2 production.[54,55] *Z. officinale* Roscoe contains 1-dehydro-6-gingerdione, 6-shogaol, 1-dehydro-10-gingerdione, and 6-dehydrogingerdione compounds which can reduce macrophage cell activation. 1-Dehydro-6-gingerdione and 6-shogaol reduce NO and COX-2 production in LPS-induced RAW 264.7 cells using Western blot assay.[56,57] 1-Dehydro-10-gingerdione reduce RAW 264.7 cells activation by reducing COX-2, IL-6, and TNF-α through NF-κB signaling pathway.[20,58] Huang *et al*.[59], reported that 6-dehydrogingerdione decreases NO, TNF-α, IL-1β, IL-6, and COX-2 via NF-κB signaling pathway. However, 6-dehydrogingerdione increases the expression of p-STAT1/p-STAT3 in LPS-induced RAW 264.7 cells.[59] In addition, *Z. officinale* Roscoe contains polysaccharide which increases NO, TNF-α, IL-1β, IL-6 production, and phagocytosis activity in LPS-induced RAW 264.7 cells.[60]

Assays

Cell viability

MTT assay is the preferred method for cell viability measurement when cell death has already occurred. In addition, the MTT assay is preferred to evaluate the effect of samples on cell viability, especially the insensitivity of toxicity detection, compared with the lactate dehydrogenase (LDH) leakage assay.[61] Interestingly, several studies used LPS to stimulate macrophage cells for the cell viability assay. Previous studies reported that LPS-activated RAW 264.7 might have decreased cell viability; however, increases or changes in cell viability have also been reported.[9] A study reported that 2 µg/mL of LPS for 24 h[62] and 5 ng/mL for 24 h[63] had no cytotoxic effects on RAW 264.7 cells. Meanwhile, a concentration of 1 µg/mL LPS was recommended for the experiment, which can activate macrophages and inhibit < 50% of cell growth[64] for 24 h.[19]

NO production

NO is synthesized from arginine by nitric oxide synthase (NOS), modulates the growth of micro-organisms, and regulates cell physiology and function.[28] The Griess method has been applied widely for determining nitric oxide levels. The Griess reagent contains equal volumes of Griess A (1% sulfanilamide in 5% phosphoric acid) and Griess B (0.1% N-1-naphthylethylendiaminedihydrochloride). The chromophore is formed by diazotization through the reaction of nitrite with sulphanilamide and coupling with naphthylethylendiaminedihydrochloride.[65] This method of NO quantification has the advantages of being quick and inexpensive and can be used in molecular biology.[40]

Cytokine

Cytokines are small protein molecules with regulatory functions in immune and inflammatory responses. Cytokines are crucial for innate and adaptive immunity, cell growth, and differentiation. Cytokines function by interacting with receptors that can be classified into several families.[48] Several methods have been developed to investigate immune responses, including determining cytokine levels in a cell culture medium. Enzyme-linked immunosorbent assay (ELISA) is used to investigate samples' effects on cytokine levels.

On the other hand, the gene expression of cytokines is determined using real-time quantitative polymerase chain reaction (qPCR) or the reverse transcription PCR method. Real-time qPCR is a highly sensitive, specific, and rapid *in vitro* method that provides both qualitative and quantitative data.[66]

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Table 1: The modulation effect and assay of RAW 264.7 cells of Zingiberaceae

No.	Plants	Extract/fraction /active compound	Induction	The modulation effect	Essay				Ref.
					Cell viability	NO production	Cytokines	Phagocytosis	
1.	*Alpinia galanga* (L). Willd. "lengkuas"	Hydroalcoholic extract	LPS 1 µg/mL	↓ NO, TNF-α, IL-6, COX-2, NF-κB, JAK/STAT, TLR-4, ↑ IL-10	MTT assay	NO (Griess reaction), iNOS (WB)	TNF-α, IL-6, IL-10 (ELISA), COX-2 (WB)		[25]
2.	*Amomum compactum* Sol. ex Maton "kapulaga"	Ethanolic extract	LPS 1 µg/mL	↓ NO, TNF-α, IL-6, COX-2, NF-κB	CCK-8 assay	NO (Griess reaction), iNOS (immunofluorescence, WB)	TNF-α, IL-6 (ELISA), COX-2 (immunofluorescence, WB)		[35]
3.	*Boesenbergia rotunda* (L.) Mansf "temu kunci"	Chalcone	IFN-γ 100 U/mL, LPS 5 µg/mL	↓ NO	MTT assay	NO (Griess reaction)			[37]
4.	*Curcuma aeruginosa* Roxb. "temu ireng"	Superoxide dismutase purified	LPS 100 ng/ml, rmIFN-γ 10 ng/ml	↓ NO	MTT assay	NO (Griess reaction)			[38]
		Ethanol extract	LPS 2 ug/mL	↓ NO	MTT assay	NO (Griess reaction)			[33]
5.	*Curcuma longa* L. "kunyit"	Aqueous extract, polysaccharide fraction	LPS 1 µg/mL	↑ NO		NO (Griess reaction)			[41]
		n-butanol fraction of aerial part	LPS 1 µg/mL	↓ NO, iNOS, TNF-α, IL-6, COX-2, NF-κB	CCK-8 assay	NO (Griess reaction), iNOS (WB)	TNF-α, IL-6 (ELISA), COX-2 (WB)		[46]
		Aqueous extract	LPS 100 ng/mL	↑ NO		NO (Griess reaction)			[42]
		Bisabolane-type sesquiterpenoid	LPS 1 µg/mL	↓ NO		NO (Griess reaction)			[69,70]
		Compounds from the water extract	LPS 20 ng/mL	↓ NO		NO (Griess reaction)			[43]
		Turmeronol A and turmeronol B	LPS 20 ng/mL	↓ NO, TNF-α, IL-1β, IL-6, NF-κB		NO (Griess reaction), iNOS (RT-PCR)	TNF-α, IL-1β, IL-6 (ELISA, RT-PCR), COX-2 (RT-PCR)		[47]
		Propylene glycol extract	*Streptococcus mutans*	↓infected cells, ↑ NO, phagocytosis	Neutral red assay	NO (Griess reaction)		Giemsa staining	[44]

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		Propylene glycol extract	*Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*	↓infected cells, ↑ NO, phagocytosis, IL-1β, TNF-α, IL-10	Neutral red assay	NO (Griess reaction)	TNF-α, IL-1β, IL-10 (ELISA)	Giemsa staining	45
6.	*Curcuma mangga* Val. & Zijp "temu mangga"	Ethanolic extract	LPS 1 µg/mL	↓ IL-6, IL-1β, TNF-α	MTT assay		IL-6, IL-1β, TNF-α (RT-PCR)		48
7.	*Curcuma zanthorrhiza* Roxb. "temulawak"	Bisdemethoxycurcumin, demethoxycurcumin, 3,3'-bis(7,7'-hydroxy-6,6'-methoxyphenyl)-Penta-(3E,2'E)-3,2'-dien-1-one, curcumin	LPS 1 µg/mL	↓ NO	MTT assay	NO (Griess reaction)			36
8.	*Curcuma zedoaria* (Christm.) Roscoe "temu putih"	Methanol extract, curcuzedoalide	LPS 1 µg/mL	↓ NO, COX-2	MTT assay	NO (Griess reaction), iNOS (WB)	COX-2 (WB)		49
		Guaiane sesquiterpene lactone (chloroform extract)	LPS 1 µg/mL	↓ NO, COX-2, TNF-α	MTT assay	NO (Griess reaction), iNOS (RT-PCR)	TNF-α, COX-2 (RT-PCR)		50
		Oil content	*Staphylococcus aureus, Listeria monocytogenes*	↓ TNF-α			TNF-α (ELISA)		71
9.	*Kaempferia galanga* L. "kencur"	Diarylheptanoid	LPS 1 µg/mL	↓ NO	MTT and LDH assay	NO (Griess reaction)			52
		Isopimarane diterpenoid	LPS 1 µg/mL	↓ NO, TNF-α, COX-2	MTT assay	NO (Griess reaction), iNOS (RT-PCR)	TNF-α (ELISA, RT-PCR), COX-2 (RT-PCR)		51
10.	*Zingiber officinale* Roscoe "jahe"	1-Dehydro-6-gingerdione, 6-shogaol	LPS 0.5 µg/mL	↓ NO, COX-2	MTT assay	NO (Griess reaction), iNOS (WB)	COX-2 (WB)		56,57
		1-Dehydro-10-gingerdione	LPS 1 µg/mL or TNFSF11 40 ng/mL	↓ COX-2, IL-6, NF-κB	WST-1 assay	iNOS (RT-PCR, WB, luciferase reporter assay)	COX-2 (WB, RT-PCR, luciferase reporter assay), IL-6 (RT-PCR, ELISA)		58
		1-Dehydro-10-gingerdione	LPS 15 and 30 µg/mL	↓ NF-κB, AP1, IRF3, TNF-α	MTT assay		TNF-α (ELISA, luciferase reporter assay)		20

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		6-Dehydrogingerdione	LPS 100 ng/mL	↓ NO, TNF-α, IL-1β, IL-6, COX-2, NF-κB, ↑ p-STAT1/p-STAT3		NO (Griess reaction), iNOS (RT-PCR, WB)	TNF-α, IL-1β, IL-6 (ELISA), COX-2 (RT-PCR, WB)		59
		Ethanolic extract	LPS 1 µg/mL	↓ NO, TNF-α, IL-1β, IL-6, COX-2	MTS assay	NO (colorimetric)	TNF-α, IL-1β, IL-6, COX-2 (ELISA)		54
		Ethanolic extract	LPS 10 ng/ml	↓ NO		NO (Griess reaction)			55
		Polysaccharide	LPS 1 µg/mL	↑ NO, phagocytosis, TNF-α, IL-1β, IL-6	MTS assay	NO (Griess reaction)	TNF-α, IL-1β, IL-6 (ELISA)	Neutral red assay	60
11.	*Zingiber officinale* var. Rubra "jahe merah"	Sun-, oven-, and freeze-dried extract	LPS 1 µg/mL	↓ NO	MTT assay	NO (Griess reaction)			39

Note: ↑(increase), ↓(decrease), 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), enzyme-linked immunosorbent (ELISA), Western blot (WB), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-sulfonyl)-2H-tetrazolium (WST-1), cell counting kit-8 (CCK-8), reverse transcriptase-polymerase chain reaction (RT-PCR), (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS)

Phagocytosis

In the innate response, activated macrophages can eliminate pathogens through direct phagocytosis.[67] A neutral red uptake assay was performed to evaluate the phagocytosis mechanism. Neutral red for live immune cells imaging of the phagocytic activity after exposure of LPS. RAW 264.7 cells were cultured and incubated with samples with or without LPS (1 µg/mL) for 24 h.[68]

Conclusion

Immunomodulators remain a topic of interest due to the continued existence of immune diseases. The Zingiberaceae family plays a role in immunomodulatory activity by regulating RAW 264.7 cell activation. Various techniques have been used to analyze the activation of macrophages in Zingiberaceae, including the MTT assay, Griess reaction, ELISA, qPCR, and neutral red uptake assay. These assays are used to determine cell viability, NO production, cytokine protein expression, gene expression, and phagocytosis activity, respectively.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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