Current Trends in Biotechnology and Pharmacy
Vol. 17 (4A) 121 - 130, October 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.4s.99

Effects of *Mitragyna Speciosa* (Korth.) on macrophage immune responses

Anwar S. K. Kafo[1], Rabia M. Elsalami[2], Nur Fatin Z. Zailan[1], Hasni Mahayidin[1], Rajesh Ramasamy[1], Uswatun H. Zaidan[3], and Masriana Hassan[1*]

[1] Department of Pathology, Faculty of Medicine and Health Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[2] Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Cyberjaya, 63000 Cyberjaya, Malaysia

[3] Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author masriana@upm.edu.my

Abstract

Mitragyna Speciosa (Korth.) or Kratom is an Asian tropical plant that exhibits medicinal benefits, including antioxidants, antibacterial, and antinociceptive effects. This study aimed to explore the immunomodulatory effect of *M. speciosa* methanolic extract (MSME) on peritoneal isolated primary macrophages (pMø) and murine-derived macrophages, RAW264.7 cell line. The cytotoxicity effect of MSME on both RAW264.7 cells and pMø was determined by a cell viability test using a Cell-Titer Blue assay. Furthermore, the effects of MSME on the secretion and expression of mediators of the immune system, including nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cytokines in lipopolysaccharide (LPS)-stimulated macrophages were evaluated by Griess assay and quantitative reverse transcription polymerase chain reaction (qRT-PCR). In addition, the effect of MSME on the phagocytic activity of LPS-stimulated macrophages was determined by a neutral red uptake assay. The cytotoxicity analysis shows stable cell viability above 80% except when treated at a high MSME concentration (>100 µg/ml). MSME exhibited anti-inflammatory effects in LPS-stimulated macrophages, as shown by a significant inhibition of NO secretion and the expression of iNOS, Tumor necrosis factor alpha (TNF-α), Interleukin-1 beta (IL-1β), and Interleukin-6 (IL-6). Furthermore, the extract also significantly reduced the phagocytic capacity of both LPS-stimulated RAW264.7 cells (36.71±6.46; MSME 100 ug/mL; p=0.0266) and pMø (42.76±10.18%; MSME 10µg/mL; p=0.0142). In conclusion, our findings suggest that treatment of MSME in LPS-activated macrophage attenuated immune responses through inhibition of phagocytic ability, reduction of NO and iNOS levels, and downregulation of TNF-α, IL-1β, and IL-6 gene expression.

Keywords: *Mitragyna speciosa*; Macrophages; Anti-inflammatory; Nitric Oxide; Cytokine.

Introduction

Kratom or scientifically known as *Mitragyna speciosa* (*M. speciosa*), is a plant belonging to the Rubiaceae family. This plant serves various purposes in conventional uses focused on easing muscle pain, reducing fever, treating wounds, managing diarrhea, alleviating coughs, and addressing hypertension. In traditional practices, the infusion of *M. speciosa* leaves acts as a tonic and is utilized as an opium or morphine substitute in folk medicine (1). *M. speciosa* has been investigated for its potential anti-inflammatory (1), antioxidant (2,3), antibacterial (2,4), anti-diabetic (3), anti-depressant (5), anti-pain, analgesic (5–7), and antipyretic effects (8). These findings revealed the versatility of *M. speciosa* as a natural remedy for

multiple ailments. A plethora of bioactive phytochemicals have been identified from the *M. speciosa* leaves, including alkaloids (e.g. mitragynine, speciogynine, paynantheine, and 7-hydroxymitragynine), polyphenol (e.g. chlorogenic acid), as well as flavonoids (e.g. epicatechin, rutin, and quercetin) (3,9–11).

M. speciosa appears to be a promising source of natural anti-inflammatory agents (1,12). The role of *M. speciosa* extracts as an anti-inflammatory agent was investigated in a carrageenan-induced paw edema animal model which has shown a significant reduction in paw edema measurement and cotton pellet-induced granulomatous tissue formation in rats (12,13). However, there is a lack of evidence to support the role of *M. speciosa* extracts on the macrophage immune responses.

Macrophages are key players in inflammatory responses due to their ability in antigen presentation, phagocytosis, and immune modulation (14). The role of macrophages in mediating the inflammatory process is through the production of various immune mediators, including cytokines, reactive oxygen species (ROS) and nitric oxide (NO) (12). The inflammatory microenvironment is a hallmark of various diseases-associated chronic inflammation. Overexpression of these immune mediators contributes to various conditions, such as chronic inflammation and tissue damage (15). Thus, inhibition of macrophage activation and their derived mediators could be a strategy for dampening inflammation and promoting tissue repair. In this present study, we evaluated the immunomodulatory properties of *M. speciosa* methanolic extract (MSME) on both peritoneal macrophages (pMø) and macrophage cell lines (RAW264.7).

Material and Methods

Plant identification and compound extraction

M. speciosa leaves were collected from Kedah, Malaysia, and the plant sample (KM 0024/22) was identified by the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). The extraction of MSME was performed by the Soxhlet method (3). The dried yield extract was dissolved in methanol and stored at -20^{o}C for further use.

Macrophage RAW264.7 cell line culture

Murine macrophage (RAW 264.7) cell line was obtained from American Type Culture Collection (ATCC® TIB-71™). The cells were maintained in a DMEM complete media (DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) at 37°C, 5% CO_2 incubator.

Peritoneal macrophage (pMø) isolation and analysis by F4/80 surface staining

This study protocol was in accordance with the Institutional Animal Care and Use Committee (IACUC) UPM/IACUC/AUP-R060/2022. The female 6–8 weeks old specific pathogen free (SPF) Balb/c mice were isolated and acclimatized for at least 7 days at the animal house of the Faculty of Medicine and Health Sciences, UPM. The isolation of pMø was performed as described previously with some modifications (16). Using a syringe, 10 mL of cold DMEM medium was injected into the peritoneal cavity of euthanized mice. After a gentle massage, pMø accumulated in the medium was collected and centrifuged (1800rpm) for 10 minutes at 4°C. The cell pellet was resuspended in a complete DMEM media containing 50 µM 2-mercaptoethanol and allowed to adhere in the cultre flask for 6 hours. The non-adherent cells were removed, and the adherent cells were cultured in a DMEM complete media at 37^{o}C, 5% CO_2 incubator for further experiments. The pMø was stained with a surface marker of Alexa Fluor® 488 conjugated monoclonal anti-mouse F4/80 (Biolegand, San Diego, CA) or IgG3 κ isotype control antibody to determine the percentage of macrophage population in the culture by Flow cytometry BD LSRFortessa™. The flow cytometry analysis indicated that 96.5% of the cells are of F4/80 positive compared to the isotype control (data not shown).

LPS stimulation and MSME treatment

Activation of macrophages was induced by LPS (E. coli 0111:B4, Merck, Germany) at 1 µg/mL for 24 hours in a complete DMEM medium and co-treated with MSME or positive control, dexamethasone (Solarbio, China) at the appropriate concentration.

Cell viability test

The cytotoxicity of MSME was determined by a CellTitre-Blue (Promega, Germany) assay according to the instructions of the manufacturer. RAW264.7 ($4x10^4$ cell/well) and pMø ($1x10^5$ cell/well) cells were seeded individually in 96-well plates overnight. The RAW264.7 and pMø were pre-incubated with MSME at serial concentrations (25, 50, and 100µg/ml) and (0.1, 1, and 10µg/ml) respictevly, for 24, 48, and 72 hours, followed by addition of 20 µL of Cell-Titre Blue solution. The plates were then further incubated for 4 hours at 37°C prior to fluorescence measurement on a FlexStation III plate reader (emission: 560 nm; excitation: 590 nm).

Griess assay

The Griess reagent was used to measure the NO production from macrophages. The $2x10^5$ cells/mL of macrophages were seeded into a 24-well plate overnight prior to 24h treatment with MSME (25, 50, and 100µg/ml in RAW264.7 cells or 0.1, 1, and 10µg/ml in pMø) or dexamethasone (10µM), in the presence or absence of LPS(1µg/ml). A 50µL/sample of cell supernatant from cultured cells (with or without LPS and/or MSME was collected and incorporated with equal volume of Griess solution (1% sulfanilamide and 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid). The solution mixtures were then incubated for 15 minutes followed by absorbance reading on a microplate reader (Dynex MRX Magellan Biosciences, USA) at 540 nm. The NO level was determined by the sodium nitrite ($NaNO_2$) standard curve (17).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted by TRIzol reagent (Invitrogen) following a manufacturer's protocol. Reverse transcription to cDNA was performed using HiScript III First Strand cDNA Synthesis Kit (Vazyme, China). The qPCR with primers of targeted genes of interest (Table 1) was performed using SYBR Green qPCR master mix (Biosystems, UK). Data were quantified using the $^{\Delta\Delta}CT$ followed by $2^{-\Delta\Delta CT}$ to quantify the relative fold change in the gene expression. The results of each sample were expressed as a $\log_2$ fold-change in gene expression (18).

Phagocytosis test

The effect of MSME on phagocytic capacity on RAW264.7 and pMø was determined by a neutral red uptake assay as described previously with slight modification (19). The $2x10^5$ cells/mL of macrophages were seeded into a 96-well plate overnight prior to co-treatment with MSME, dexamethasone, and/or LPS, as described in the previous assay. Following 24 hours of incubation, the cells were preloaded with 100 µL of neutral red solution (0.075%) for 30 minutes at room temperature. Then the solution in the plate was discarded, and the cells were rinsed with PBS to remove any non-phagocytized neutral red before the addition of 200 mL of cell lysis buffer

Table 1: Sequences of primers used in qRT-PCR.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
β-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
iNOS	AGACCTCAACAGAGCCCTCA	TCGAAGGTGAGCTGAACGAG
IL-1β	AACTCAACTGTGAAATGCCACC	CATCAGGACAGCCCAGGTC
IL-6	GCCTTCTTGGGACTGATGCT	GACAGGTCTGTTGGGAGTGG
TNF-α	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT

iNOS, inducible nitric oxide synthase; IL, interleukin; TNF-α, tumour necrosis factor-α

($V_{ethanol}$: $V_{acetic\ acid}$ =1:1). The optical density was measured using a microplate reader (Dynex Mrx Magellan Biosciences, USA) at 540 nm.

Statistical analysis

Data from the findings were analyzed using GraphPad Prism software (version 9.0). A one-way analysis of variance (ANOVA) with Tukey's post hoc test was used to compare different treatment groups and control. Furthermore, analysis by a two-way ANOVA was used to compare two groups (stimulated and non-stimulated cells). The data were expressed as the mean ± standard error of the mean (SEM) of two or three independent experiments.

Results and Discussion

Effect of MSME on the viability of macrophages

The analysis of cytotoxicity levels of MSME is crucial to determine the workable concentration of the extract in the cell culture. In the present study, we explored the effects of MSME on inflammatory mediators and phagocytic activity of LPS-stimulated macrophages. We first assessed the cytotoxicity of MSME on both RAW264.7 and pMø macrophages through determination of the cell viability to exclude any probable cytotoxic effects. Our data showed that the viability of both RAW264.7 and pMø significantly reduced at 100 µg/mL MSME and deteriorated by approximately 50% at higher (>250 µg/mL) MSME concentrations (Figure 1).

The inhibitory concentration of 20% (IC_{20}) of cells by MSME was indicated at 223.2±10.96 µg/mL and 19.84±4.56 µg/mL in RAW264.7 and pMø, respectively. This finding suggests that MSME at concentrations below 100µg/mL did not show any cytotoxic effects on RAW264.7 cells. On the other hand, the toxicity effect of MSME on pMø was shown at a concentration of ≥25 µg/mL. Therefore, in the subsequent experiment, the working concentrations of MSME were arranged at 25, 50, and 100 µg/mL for the RAW264.7 cell line and 0.1, 1, and 10 µg/mL for pMø to ensure the viability of cells reached above 80% in MSME treatment. In a previous related study, *M. speciosa* showed some toxicity effects on several different types of cell lines (i.e. HEK 293, HepG2, MCL-5, SH-SY5Y, and cHol cell lines) when treated at ≥100 µg/mL [20]. In addition, the cytotoxicity effects of the extract were also investigated in CaCO2 and SH-SY5Y cell lines which indicated toxicity level at ≥60 µg/mL (21).

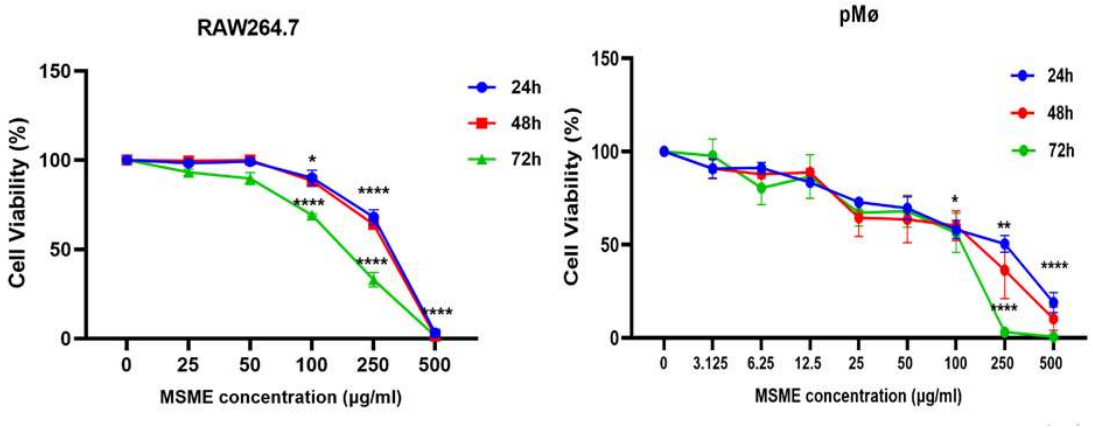

Figure 1: Cytotoxicity effect of MSME on macrophages. Both RAW264.7 and pMø were cultured individually in 96-well plates and treated with MSME (0 to 500 µg/mL) for 24, 48 and72 hours. Values are mean ±SEM of three independent experiments. *p<0.05, **p<0.001

MSME inhibits LPS-induced NO production

NO is one of the main mediators produced by phagocytes that contribute to both physiological and pathological processes of inflammation induced by the activation of inducible nitric oxide synthase (iNOS). In response to bacterial endotoxin, macrophages also induce the activation of host defence mechanisms via the production of some immune mediators, including NO and pro-inflammatory cytokines (14). The effect of MSME on the secretion of NO in both RAW264.7 and pMø was determined by the production of nitrite in the cell culture supernatant in comparison with dexamethasone (Figure 2). Stimulation of LPS in macrophages promotes large amounts of NO secretion. The production of NO in LPS-stimulated macrophages significantly increased from 1.28±0.53 µM to 16.79±0.73 µM; p<0.0001 in RAW264.7 cells and 1.48±0.11 µM to 3.97±0.30 µM; p=0.0045 in pMø. Dexamethasone, as expected, greatly decreased the production of NO to 3.92±0.28 µM; p<0.0001 (in RAW264.7) and 0.49±0.30 µM; p=0.0004 (in pMø). In both types of macrophages, the levels of NO were gradually reduced by MSME in a concentration-dependent manner (Table 2). For example, at the highest concentration (100 µg/mL in RAW264.7 and 10 µg/mL in pMø), MSME greatly reduced NO production from

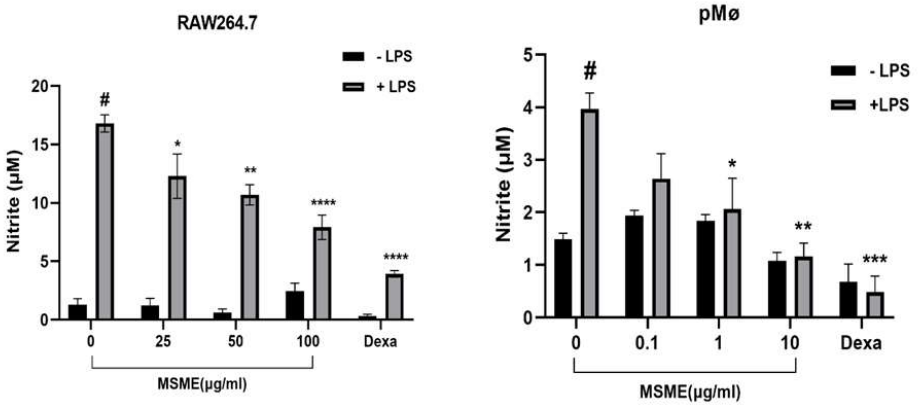

Figure 2: Inhibition of NO production in macrophages. Both RAW264.7 cells and pMø were cultured individually in 96-well plates in the presence or absence of LPS (1 µg/mL; 24 hours incubation), MSME, and/or dexamethasone (Dexa). The NO levels were determined by measuring the amount of nitrite by Griess assay. Values are mean ±SEM of three independent experiments. #p<0.05 indicates statistically significant in comparison with the unstimulated control. *p<0.05, **p<0.001, and ***p<0.0001 indicate statistically significant in comparison with the LPS stimulation without MSME treatment.

Table 2: The effects of MSME on NO production in LPS-stimulated macrophages.

RAW264.7			pMø		
MSME (ug/mL)	**NO inhibition (%)**	***P* value**	**MSME (ug/mL)**	**NO inhibition (%)**	***P* value**
25	26.8	0.0285	0.1	33.4	0.2027
50	36.3	0.0011	1	47.9	0.0324
100	52.9	<0.0001	10	70.6	0.0022
Dexa	76.6	<0.0001	Dexa	87.6	0.0004

NO, nitric oxide; MSME, *Mitragyna speciosa* methanolic extract; pMø, peritoneal macrophages.

16.79±0.73 µM to 7.90±1.04 µM; p<0.0001 and 3.97±0.30 µM to 1.16±0.25 µM; p=0.0022, respectively. Our results have shown that MSME significantly decreased NO production, which was previously increased by LPS stimulation. In addition, the positive control, dexamethasone, had stronger effects compared to MSME in reducing NO levels. Our data was in line with a study conducted by Tohar et al., 2016, which demonstrated a moderate inhibitory activity in preventing the generation of NO by *M. speciosa* active compound (1).

Effect of MSME on LPS-induced iNOS and cytokine gene expression

Nitric oxide synthases (NOS) are a group of enzymes that are responsible for the production of NO in mammalian cells (22). iNOS is one of the NOS isoforms that is primarily involved in the pathophysiology of inflammation through the secretion of NO in response to bacterial LPS or IFN-γ (14). In addition, those stimulations also activate the transcription factor of NFκB and initiate the generation of cytokines which act as mediators of inflammation (23). To the best of our knowledge, no other research has evaluated the expression of iNOS and pro-inflammatory cytokines in macrophages exposed to *M. speciosa extracts*. In the present study, the expression of iNOS, TNF-α, IL-1β, and IL-6 genes in RAW264.7 cells was determined by qRT-PCR. As shown in Figure 3, the stimulation of RAW264.7 cells with LPS alone significantly increased the expression levels of iNOS (p=0.0112), TNF-α (p=0.0371), IL-1β (p=0.0012), and IL-6 (p=0.0085). On the other

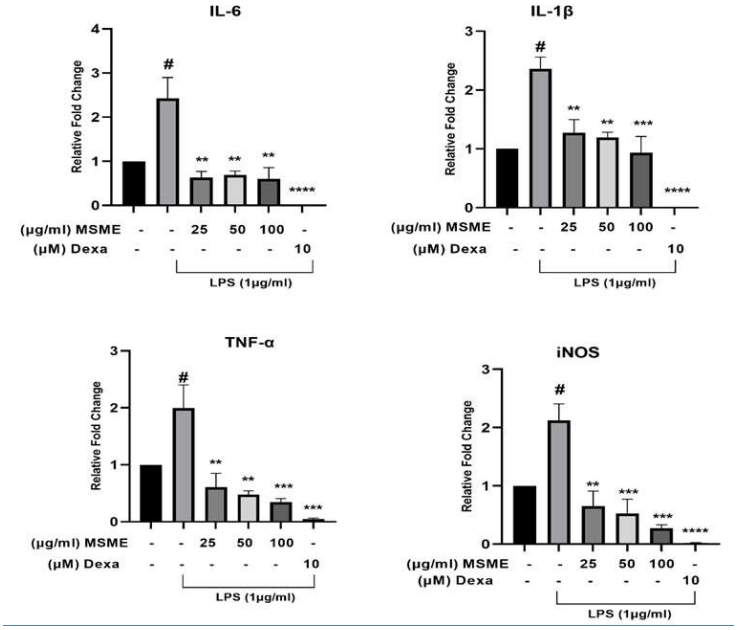

Figure 2: Expression of pro-inflammatory cytokines and mediators secreted by the MSME-treated LPS-stimulated RAW264.7 cells. Total mRNA was isolated from RAW264.7 cells that were cultured in the presence or absence of LPS (1 µg/mL; 24 hours incubation), MSME, and/or dexamethasone (Dexa). The expression of iNOS, TNF-α, IL-1β, and IL-6 were quantified by qRT-PCR. All data were normalized with the housekeeping gene (β-actin). Values are mean ±SEM of three independent experiments. #p<0.05 indicates statistically significant in comparison with the unstimulated control. *p<0.05, **p<0.001, and ***p<0.0001 indicate statistically significant in comparison with the LPS stimulation without MSME treatment.

hand, treatment of the LPS-stimulated cells with MSME reduced the expression of iNOS, TNF-α, IL-1β, and IL-6 genes of RAW264.7 cells in all concentrations tested. Cytokines, typically TNF-α, IL-1β, and IL-6, are mainly involved in inflammation and are associated with the development of many chronic inflammatory diseases, including obesity, diabetes mellitus, and hypertension (24,25). Inhibition of these inflammatory cytokines was found to attenuate some disease-related conditions associated with inflammation by reducing inflammatory components leading to improvement of vascular function. This strategy has been explored in inflammatory diseases such as type 2 diabetes and hypertension (24–26). In our present study, MSME had shown potential anti-inflammatory activities through its strong capacity to reduce the expression of pro-inflammatory cytokines in macrophages. This suggests the potential of *M. speciosa* in the alleviation of inflammatory conditions. In addition, the inhibition of NO production by MSME that was previously investigated may be associated with reduced iNOS gene expression in macrophages. The correlation between the production of NO and iNOS gene expression was investigated in other compounds, such as *Tribulus terrestris* L. and *Pyrolae herbal* extracts. These extracts have shown the ability to inhibit NO production due to the downregulation of iNOS expression (27,28).

Influence of MSME on macrophage phagocytic capacity

Activation of inflammatory signals in macrophages induces phagocytosis that primarily promotes the elimination of pathogens and cell debris. On the other hand, negative regulation of macrophage activation is essential for homeostasis and termination of immune and inflammatory responses (29), which might be beneficial to prevent phagocyte-mediated xenograft rejection (30). In this study, the effect of MSME on the phagocytic activity of macrophages was evaluated by neutral red uptake assay. The macrophage cell was exposed to different concentrations of MSME with or without LPS. Figure 4 shows that the MSME has no significant effect on the un-stimulated

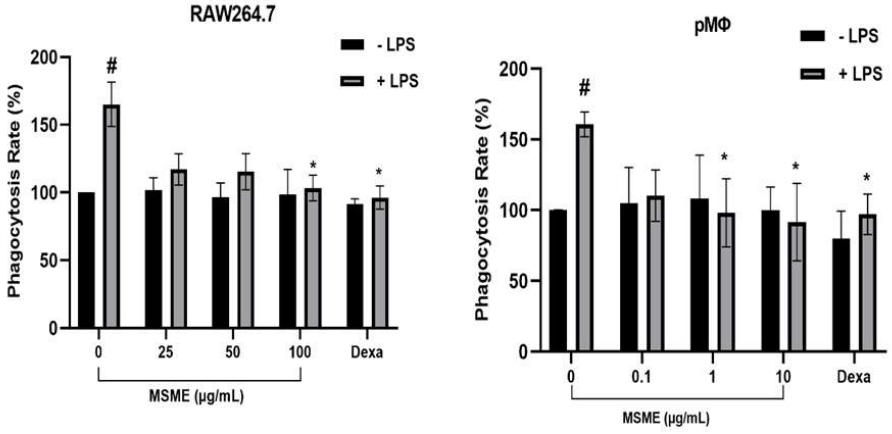

Figure 4: Effect of MSME on phagocytic activity of macrophages. Both RAW264.7 cells and pMø were cultured individually in 96-well plates in the presence or absence of LPS (1 µg/mL; 24 hours incubation), MSME, and/or dexamethasone (Dexa). The phagocytosis was determined by measuring the optical density of lysed cells. Values are mean ±SEM of three independent experiments. #$p<0.05$ indicates statistically significant in comparison with the unstimulated control. *$p<0.05$ indicates statistically significant in comparison with the LPS stimulation without MSME treatment.

macrophage. On the other hand, the elevation of phagocytic activity of LPS-stimulated macrophages was significantly inhibited by MSME and dexamethasone. This finding might be associated with the downregulation of inflammatory mediators by MSME, which is presented by reduced NO production and downregulation of iNOS and cytokine expression. Several immunosuppressive plants have been reported, including *Moringa oleifera* seed extract, which reduced carbon particle-induced phagocytosis in Swiss albino mice (31). Similarly, extracts from *Zingiber zerumbet* showed inhibition of phagocytic activity of neutrophils isolated from a whole Wistar rat blood (32).

Conclusion

In conclusion, MSME has potential anti-inflammatory properties and possesses immunosuppressive activities presented by its capacity to modulate the inflammatory responses in LPS-activated macrophages through the reduction of NO levels and iNOS, TNF-α, IL-6, and IL-1β gene expression. In addition, the decline in macrophage phagocytic activity might be attributed to the suppression of the inflammatory responses by MSME.

Acknowledgement

This study was financially supported by the Universiti Putra Malaysia (UPM) through the Geran Putra Siswazah (GP-IPS/2022/9726100).

Conflict of Interest

The authors declare no conflict of interest.

References

1. Tohar, N., Shilpi, J. A., Sivasothy, Y., Ahmad, S. and Awang, K. (2016) Chemical constituents and nitric oxide inhibitory activity of supercritical carbon dioxide extracts from Mitragyna speciosa leaves. *Arab. J. Chem.* 12: 350–359.

2. Parthasarathy, S., Azizi, J. B., Ramanathan, S., Ismail, S., Sasidharan., Said, M. I. M. and Mansor, S. M. (2009) Evaluation of antioxidant and antibacterial activities of aqueous, methanolic and alkaloid extracts from Mitragyna speciosa (rubiaceae family) leaves. *Molecules* 14, 3964–3974.

3. Zailan, N. F., Seri Narti, S. and Hassan, M. (2022) Evaluation of Phytochemical Composition , Antioxidant and anti- Diabetic Activities of Mitragyna speciosa Methanolic Extract (MSME). *Malaysian J. Med. Heal. Sci.* 18, 92–99.

4. Juanda, E., Andayani, S. and Maftuch, M. (2019) Phytochemical Screening and Antibacterial Activity of Kratom Leaf (Mitragyna speciosa Korth.) Against Aeromonas hydrophilla. *J. Exp. Life Sci.* 9, 155–158.

5. Buckhalter, S., Soubeyrand, E., Ferrone, S. A. E., Rasmussen, D. J., Manduca, J. D., Al-Abdul-Wahid, M. S., Frie, J. A., Khokhar, J. Y., Akhtar, T. A. and Perreault, M. L. (2021) The Antidepressant-Like and Analgesic Effects of Kratom Alkaloids are accompanied by Changes in Low Frequency Oscillations but not ΔFosB Accumulation. *Front. Pharmacol.* 12, 1–20.

6. Vicknasingam, B., Tink, W., Abdul, A. and Ramachandram, D. (2020) Kratom and Pain Tolerance : A Randomized ,. *Yale J. Biol. Med.* 93, 229–238.

7. Kruegel, A. C., Uprety, R., Grinnell, S. J., Langreck, C., Pekarskaya, E. A., Rouzic, V. L., Ansonoff, M., Gassaway, M. M., Pintar, J. E., Pasternak, G. W., Javitch, J. A., Majumdar, S. and Sames, D. (2019) 7-Hydroxymitragynine Is an Active Metabolite of Mitragynine and a Key Mediator of Its Analgesic Effects. *ACS Cent. Sci.* 5, 992–1001.

8. Annas, S., Mossadeq, W. M. S. and Kadir, A. A. (2020) Antipyretic effect of mitragynine and crude methanolic extract of mitragyna speciosa korth. In mice. *Pertanika J. Trop. Agric. Sci.* 43, 207–216.

9. Firmansyah, A., Sundalian, M. and Taufiq, M. (2021) Kratom (Mitragyna speciosa korth) for a new medicinal: A review of pharmacological

and compound analysis. *Biointerface Res. Appl. Chem.* 11, 9704–9718.

10. Hassan, Z., Muzaimi, M., Navaratnam, V., Yusoff, N. H. M., Suhaimi, F. W., Vadivelu, R., Vicknasingam, B. K., Amato, D., Hörsten, S. V., Ismail, N. I. W., Jayabalan, N., Hazim, A. I., Mansor, S. M. and Müller, C. P. (2013) From Kratom to mitragynine and its derivatives: Physiological and behavioural effects related to use, abuse, and addiction. *Neurosci. Biobehav. Rev.* 37, 138–151.

11. Pantano, F., Tittarelli, R., Mannocchi, G., Zaami, S., Ricci, S., Giorgetti, R., Terranova, D., Busardò, F. P. and Marinelli, E. (2016) Hepatotoxicity induced by “the 3Ks”: Kava, Kratom and khat. *Int. J. Mol. Sci.* 17.

12. Mossadeq, S. W. M., Sulaiman, M.R.,Tengku Mohamad, T.A., Chiong, H.S., Zakaria, Z.A., Jabit, M.L., Baharuldin, M.T.H. and Israf, D.A. (2009) Anti-inflammatory and antinociceptive effects of Mitragyna speciosa Korth methanolic extract. *Med. Princ. Pract.* 18, 378–384.

13. Saleh, S. R., Hasan, M. H., Said, M. I. M., Adenan, M. I. and Adam, A. (2012) Antioxidant, anti-inflammatory and antinociceptive activities of Mitragyna speciosa and Erythroxylum cuneatum. *SHUSER 2012 - 2012 IEEE Symp. Humanit. Sci. Eng. Res.* 1087–1091.

14. Moro, C., Palacios, I., Lozano, M., D’Arrigo, M., Guillamón, E., Villares, A., Martínez, J. A. and Lafuente, A. G. (2012) Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages. *Food Chem.* 130, 350–355.

15. Yoon, S. B., Lee, Y. J., Park, S. K., Kim, H., Bae, H., Kim, H. M., Ko, S. G., Choi., H. Y., Oh, M. S. and Park, W. Anti-inflammatory effects of Scutellaria baicalensis water extract on LPS-activated RAW 264.7 macrophages. *J. Ethnopharmacol.* 125, 286–290 (2009).

16. Alnuqaydan, A. M., Almutary, a., Bhat, G. R., Mir, T. A., Wani, S. I., Rather, M. Y., Mir, S. A., Alshehri, B., Alnasser, S., Zainy, F. M. A. and Rah, B. (2022) Evaluation of the Cytotoxic, Anti-Inflammatory, and Immunomodulatory Effects of Withaferin A (WA) against Lipopolysaccharide (LPS)-Induced Inflammation in Immune Cells Derived from BALB/c Mice. *Pharmaceutics* 14,.

17. Srisuwan, S., Tongtawe, P., Srimanote, P. and Voravuthikunchai, S. P. (2014) Rhodomyrtone modulates innate immune responses of THP-1 monocytes to assist in clearing methicillin-resistant Staphylococcus aureus. *PLoS One* 9, 1–11.

18. Liu, Y., Chen, W., Zheng, F., Yu, H. and Wei, K. (2022) Xanthatin Alleviates LPS-Induced Inflammatory Response in RAW264.7 Macrophages by Inhibiting NF-κB, MAPK and STATs Activation. *Molecules* 27.

19. Chang, Y., Guo, A., Jing, Y., Lin, J., Sun, Y., Kong, L., Zheng, H. and Deng, Y. (2021) Immunomodulatory activity of puerarin in RAW264.7 macrophages and cyclophosphamide-induced immunosuppression mice. *Immunopharmacol. Immunotoxicol.* 43, 223–229.

20. Saidin, N. A. (2008) Cytotoxicity of extract of Malaysian Mitragyna speciosa Korth and its dominant alkaloid mitragynine. (IMPERIAL COLLEGE LONDON).

21. Oliveira, A. S., Fraga, S., Carvalho, F., Arau´jo, A. M., Pereira, C. C., Teixeira, J. P., Bastos, M. D. L., Pinho, P. G. D. (2016) Chemical characterization and in vitro cyto- and genotoxicity of ‘legal high’ products containing Kratom (Mitragyna speciosa). *Forensic Toxicol.* 34, 213–226.

22. Zamora, R., Vodovotz, Y. and Billiar, T. R. (2000) Inducible nitric oxide synthase and inflammatory diseases. *Mol. Med.* 6, 347–373.

23. Gasparrini, M., Hernandez, T. Y. F., Giampieri, F., Afrin, S., Suarez, J. M. A., Mazzoni, L., Mezzetti, B., Quiles, J. L. and Battino, M. (2017) Anti-inflammatory effect of strawberry extract against LPS-induced stress in RAW 264.7 macrophages. *Food Chem. Toxicol.* 102, 1–10.

24. Tanase, D. M., Gosav, E. M., Radu, S., Ouatu, A., Rezus, C., Ciocoiu, M., Costea, C. F., Floria, M. (2019) Arterial Hypertension and Interleukins: Potential Therapeutic Target or Future Diagnostic Marker? *Int. J. Hypertens.* 2019.

25. Popko, K., gorska, E., Stelmaszczyk-Emmel, A., Plywaczewski, R., Stoklosa, A., gorecka, D., Pyrzak B. and Demkow, U. (2010) Pro-inflammatory cytokines IL-6 and TNF-α and the development of inflammation in obese subjects. *Eur. J. Med. Res.* 15, 120–122.

26. Lainampetch, J., Panprathip, P., Phosat, C., Chumpathat, N., Prangthip, P., Soonthornworasiri, N., Puduang, S., Wechjakwen, N. and Kwanbunjan, K. (2019) Association of Tumor Necrosis Factor Alpha, Interleukin 6, and C-Reactive Protein with the Risk of Developing Type 2 Diabetes: A Retrospective Cohort Study of Rural Thais. *J. Diabetes Res.* 2019, 10–12.

27. Lee, M. H., Lee, J. M., Jun, S. H., Lee, S. H., Kim, N. W., Lee, J. H., Ko, N. Y., Mun, S. H., Kim, B. K., Lim, B. O., Choi, D. K. and Choi, W. S. (2007) The anti-inflammatory effects of Pyrolae herba extract through the inhibition of the expression of inducible nitric oxide synthase (iNOS) and NO production. *J. Ethnopharmacol.* 112, 49–54.

28. Zhao, W. R. Shi, W. T., Zhang, J., Zhang, K. Y., Qing, Y., Tang, J. Y., Chen, X. L. and Zhou, Z. Y. (2021) Tribulus terrestris L. Extract Protects against Lipopolysaccharide-Induced Inflammation in RAW 264.7 Macrophage and Zebrafish via Inhibition of Akt/MAPKs and NF-B/iNOS-NO Signaling Pathways. *Evidence-based Complement. Altern. Med.* 2021.

29. Hoek, R. H., Ruuls, S. R., Murphy, C. A., Wright, G. J., Goddard, R., Zurawski, S. M., Blom, B., Homola, M. E., Streit, W. J., Brown, M. H., Barclay, A. N. and Sedgwick, J. D. (2000) Down-regulation of the macrophage lineage through interaction with OX2 (CD200). *Science (80-.).* 290, 1768–1771.

30. Wang, H., VerHalen, J., Madariaga, M. L., Xiang, S., Wang,S., Lan, P., Oldenborg, P. A., Sykes,M. and Yang, Y. G. (2007) Attenuation of phagocytosis of xenogeneic cells by manipulating CD47. *Blood* 109, 836–842.

31. Mahajan, S. G. and Mehta, A. A. (2010) Immunosuppressive activity of ethanolic extract of seeds of Moringa oleifera Lam. in experimental immune inflammation. *J. Ethnopharmacol.* 130, 183–186.

32. Ghazalee, N. S., Jantan, I., Arshad, L. and Haque, M. A. (2019) Immunosuppressive effects of the standardized extract of Zingiber zerumbet on innate immune responses in Wistar rats. *Phyther. Res.* 33, 929–938.