ORIGINAL ARTICLE

Impact of *Mitragyna speciosa* Methanolic Extract on Adaptive Immunity: Investigated in SRBC-induced Delayed-type Hypersensitivity Mouse Model

Anwar Salm Kalifa Kafo[1], Rabia Mrehil Elsalami[2], Shamima Abd Rahman[2], Rajesh Ramasamy[1], Hasni Mahayidin[1], Masriana Hassan[1]

[1] Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2] Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Cyberjaya, 63000 Cyberjaya, Selangor, Malaysia

ABSTRACT

Introduction: *Mitragyna speciosa* (Korth.) or Kratom contains several bioactive compounds that have potential therapeutic benefits. The present study investigated the potential immunomodulatory effects of *Mitragyna speciosa* methanolic extract (MSME) in delayed-type hypersensitivity (DTH) mouse model. **Materials and methods:** Female Balb/c mice were induced with DTH by sheep red blood cells (SRBCs) following the administration of MSME. The thickness of paw edema that developed following subcutaneous injection of SRBC on the right hind footpad of the mice was measured. The blood samples and spleen were collected for investigation of the effects of MSME on antibody production, complete blood count (CBC), spleen index, splenocyte proliferation, and lymphocyte (CD4, CD8, and CD19) populations. **Results:** The data demonstrated that MSME significantly reduced the paw edema induced by SRBC and showed a marked reduction in anti-SRBC antibody levels. However, no significant changes were observed in the CBC, spleen index, and CD4, CD8, and CD19 subset populations. In addition, stimulation of splenocytes isolated from MSME-treated SRBC-induced DTH mice with lipopolysaccharide (LPS) or Concanavalin A (Con A) ex vivo reduced cell proliferation. **Conclusion:** These data demonstrated that MSME potentially inhibits immune response by suppressing DTH reactions, reducing antibody production and cell proliferation without affecting the lymphocyte profiles. These findings suggest the immunomodulatory effects of MSME through immunosuppressive and anti-inflammatory activities.
Malaysian Journal of Medicine and Health Sciences (2024) 20(SUPP11): 34-40. doi:10.47836/mjmhs20.s11.6

Keywords: *Mitragyna speciosa*, Kratom, Delayed-type hypersensitivity, Immunomodulatory, CD4

Corresponding Author:
Masriana Hassan, PhD
Email: masriana@upm.edu.my
Tel :+603-97692381

INTRODUCTION

The immune system plays a crucial role in protecting the body from infections and other diseases by providing a specific, targeted immune response to harmful pathogens and foreign substances. When immune function is compromised, it can lead to a range of diseases such as immunodeficiency disorders, autoimmune diseases, and cancer. Natural compounds with immunomodulatory activities can serve as an alternative therapy for various diseases by regulating normal immunological functions. The enhancement of the immune response to better fight off infection, diseases, or cancer characterizes immunostimulation. Conversely, immunosuppression is marked by inhibition of the immune system's ability to respond against antigens and is often used to manage specific medical conditions such as autoimmune diseases [1].

Mitragyna speciosa (M. speciosa) is a plant belonging to the Rubiaceae family and it has been used in Southeast Asia for a variety of purposes, such as alleviating fatigue, enhancing physical stamina, recreational drug, and serving as a substitute for opium [2]. Its primary indole alkaloid, mitragynine, has been shown to have antipyretic, antidepressant, anxiolytic, antiviral, and antidiabetic effects [3]. Recently, the plant extract has demonstrated anti-inflammatory properties by reducing the production of nitric oxide (NO) and pro-inflammatory cytokines induced by lipopolysaccharide (LPS) in macrophages [4,5] and reducing the development of carrageenan-induced rat paw edema granulomatous tissue formation in the inflammatory mice model [6]. However, the immunomodulatory potential of *M. speciosa,* particularly in adaptive immune responses has

not yet been explored.

Bioactive compounds derived from plants have demonstrated a wide range of therapeutic benefits. Understanding the mechanisms of how these compounds modulate the host's immune response and provide primary sources of safe and effective immunomodulators is of great interest [7]. Evaluation of the immunomodulatory effects of M. speciosa extract on cellular- and humoral-mediated immune responses by either enhancing or suppressing immune activity is crucial for developing therapies for autoimmune diseases or for boosting the immune system in cases of immunodeficiency. Therefore, we aimed to evaluate the immunomodulatory potential of *M. speciosa* against sheep red blood cells (SRBCs)-induced Delayed-type hypersensitivity (DTH) in Balb/c mice.

MATERIALS AND METHODS

Mitragyna speciosa methanolic extract (MSME)

M. speciosa leaves were collected from Kedah, Malaysia, and the plant sample (denoted as KM 0024/22) underwent identification at the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). The crude extract of the leaves was isolated by methanolic extraction as described in our previous study [4]. The extract was dissolved into a 1% carboxymethyl cellulose (CMC) and stored at 4oC until needed.

Experimental animals

Female Balb/c mice (N=42), between 20 to 25 g, were obtained from the Comparative Medicine and Technology Unit (COMeT), UPM. The animals were acclimatized and maintained in a clean environment with a temperature of 25±2°C, 78±2% humidity level, and a 12-hour light/dark cycle. The mice had unrestricted access to a standard mouse pellet diet and water *ad libitum*. All animal-related procedures were conducted following the approval of the University of Cyberjaya Animal Care and Use Committee (CACUC) (CACUC/1/2023/2). The animals were equally divided into 7 groups and orally treated for 14 days as follows: (1) phosphate-buffered saline (PBS) as control; (2 to 5) 0, 25, 100, or 200 mg/kg BW of MSME; (6) Levamisole (2.5 mg/kg BW); and (7) Cyclophosphamide (30 mg/Kg BW).

Delayed-Type Hypersensitivity (DTH) mouse model

The DTH reaction was induced in all mice except the control group. Briefly, 5×10^9 cells/mL sheep red blood cells (SRBCs) were prepared in a 1:1 ratio of Alsever's solution (Sigma Aldrich, Merck, USA) according to Hamid et al [8]. The immunization was done on day 7 post-treatment through intraperitoneal injection with 200 µl of SRBC [9–11]. The baseline paw thickness of each animal was recorded. Subsequently, on day 14, the mice were challenged by subcutaneous injection into the right hind footpad with 20 µl of the same antigen [12]. The DTH assessment was conducted to elucidate the cellular immune response in the treated animal subjects. The paw thickness was measured 24 hours post-injection by a digital caliper. The edema development was quantified by calculating the difference in paw thickness from baseline to post-challenge measurements.

Complete Blood Count (CBC)

A total of 500 µl of blood sample was obtained through cardiac puncture from each mouse 24 hours post-SRBC challenge. The CBC analysis was carried out using a hematology analyser (NIHON KOHDEN, Celltac, MEK-6550, Japan) at the Faculty of Veterinary Medicine, UPM.

Hemagglutination assay

The serum was isolated through centrifugation of the blood at 2000 rpm for 5 minutes. 50 µl/well serial dilution (from 1:2 to 1:4096) of serum samples was dispensed into a round-bottomed 96-well plate. Subsequently, 50 µl of 1% v/v SRBCs in PBS was added to each well. The plates were gently shaken for 1 min and incubated at room temperature for 24 hours, while agglutination was visually monitored. The presence of anti-SRBC antibodies was determined at the highest dilution titer of the agglutination reaction.

Spleen Index

The spleen index expressed in mg per 10 g of body weight was calculated based on Yun et al., 2018, following aseptically excised and weighed [13].

$$\text{Spleen index (mg/10g)} = \frac{\text{spleen weight, mg}}{\text{body weight, g}} \times 10$$

The above formula was used to normalize spleen size based on individual body weight. This is particularly useful in studies involving animals of different sizes, where absolute spleen weight alone may not accurately reflect differences due to body weight variations. It offers a more precise measurement, especially in situations where slight variations in spleen size need to be accurately documented.

Splenocyte culture

The spleen was homogenized using a 5 ml syringe plunger and filtered through a 70 µm nylon cell strainer to obtain a uniform cell suspension. After centrifugation (800 Чg, 3 min at 4°C), the red blood cells (RBCs) were lysed using 1x RBC lysing buffer (Sigma-Aldrich™, Merck, USA) and incubated for 5 min at room temperature. Following a wash with PBS, the cells were resuspended in RPMI medium containing 1% penicillin-streptomycin, 10% FBS, and 50 µM 2-mercaptoethanol, and incubated in a 37°C, 5% CO2 incubator. These non-purified cells are known as splenocytes. Splenocytes contain various cell types including lymphocytes (T and B cells), dendritic

cells, and macrophages.

Cell proliferation assay

The proliferation of splenocytes was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [12]. Following the splenocyte isolation, the cells ($4x10^5$ cells/well) were cultured in the presence or absence of 5 µg/mL concanavalin A (Con A) or 1 µg/mL LPS for 72 hours. MTT reagent (5 mg/mL) was added to each well followed by 4 hours of incubation. Finally, 100 µL of dimethyl sulfoxide (DMSO) was added to each well and the plates were then placed in a dark at room temperature for approximately 30 minutes. The absorbance of the samples was measured at 570 nm using a microplate reader (Plate Chameleon V, Hidex, Finland). Optical density values were converted into cell proliferation percentages with the following formula [14]:

$$\text{Proliferation rate (\%)} = \frac{AS}{AC} \times 100$$

Where, As is the absorbance of test sample and Ac is absorbance of control.

Immunophenotyping

The immunophenotyping of lymphocytes was performed using fluorochrome-conjugated CD4, CD8, and CD19 antibodies through flow cytometry. The splenocytes ($1X10^6$ cells/mL) were labeled with a 1:1000 dilution of the viable Zoombie dye (BioLegend, USA, Cat:423105/423106) to discriminate dead cells. Subsequently, the cells were stained with 0.5 µg of APC-anti-mouse-CD4, 0.125 µg of PE-anti-mouse-CD19, or 0.25 µg of PerCP/cyanin-anti-mouse-CD8 antibodies (BioLegend, USA). The cell acquisition was performed by a flow cytometry instrument LSR FortessaTM cell analyser (BD Biosciences, San Jose, USA).

Statistical Analysis

Statistical analysis was conducted through a one-way or two-way analysis of variance (ANOVA), as stated in individual experiments, using the GraphPad Prism software version 9.0 (GraphPad Software Inc., USA).

RESULTS

Inhibition of the DTH reaction by MSME

The effects of MSME on the immune response were evaluated in a DTH mouse model. The baseline average paw thickness of the mice pre-SRBC challenge ranged from 1.3 mm to 2.0 mm. Following the SRBC challenge, the DTH reaction can be observed through the formation of edema on the hind footpad. Figure 1a shows representative photographs of the development of edema in each group (left) and analysis of paw thickness (right) after normalizing it with the baseline thickness (pre-SRBC challenge) in each respective animal. The graph shows a significant increase ($p<0.0001$) in edema formation post-SRBC challenge. A substantial reduction in the paw edema thickness was demonstrated in an MSME dose-dependent manner. The maximum edema inhibition was shown at the highest dose of MSME (200 mg/kg BW) in which the thickness was reduced from 0.92±0.15 mm to 0.45±0.07 mm ($p<0.0251$). The levamisole and cyclophosphamide control groups were expected to promote (0.92±0.15 mm to 1.50± 0.12 mm; $p=0.0025$) and suppress (0.92±0.15 mm to 0.28± 0.04 mm; $p=0.0009$) edema formation, respectively.

The spleen of each mouse was dissected out and weighed to evaluate if there were any changes in the spleen index. Figure 1b shows there is a marginal increase with no significant difference in MSME treatment [50 mg/kg (41.72± 3.764 mg/10g; $p= 0.6906$), 100 mg/kg (39.77±2.481 mg/10g; $p=0.9384$), and 200 mg/kg (39.37±2.309 mg/10g; $p=0.9631$)] compared to the normal control group (30.19±0.948 mg/10g) or untreated DTH (35.96±3.437 mg/10g). On the other hand, levamisole and cyclophosphamide showed a significant increase (51.91±2.66 mg/10g; $p=0.0018$) and decrease (24.61±0.85 mg/10g; $p=0.0499$) in spleen index, respectively (Figure 1b).

Serum from the blood samples of each mouse was collected to determine the production of anti-SRBC antibodies based on agglutination reactions that occur at the highest serum dilution titer. As shown in Figure 1c, the production of anti-SRBC antibodies has increased significantly in untreated DTH (96.00±14.31 titer; $p=0.0006$). However, MSME (200 mg/kg BW) reduced the production of antibodies (24.00±3.58 titer; $p=0.0167$) which was in line with cyclophosphamide (2.67±0.42 titer; $p=0.0009$). On the other hand, levamisole shows a substantial increase in antibody production (192.0±28.62 titer; $p=0.0006$).

The whole blood samples were also analyzed for CBC including red blood cells (RBC), hemoglobin (Hb), white blood cells (WBC), lymphocytes, and monocytes (Table I). The result indicates no statistically significant changes in cell count of all MSME-treated groups compared to the control group. Levamisole, however, demonstrates a significant elevation in WBC, lymphocytes, and monocytes. Conversely, cyclophosphamide is associated with a reduction in the overall blood cell count.

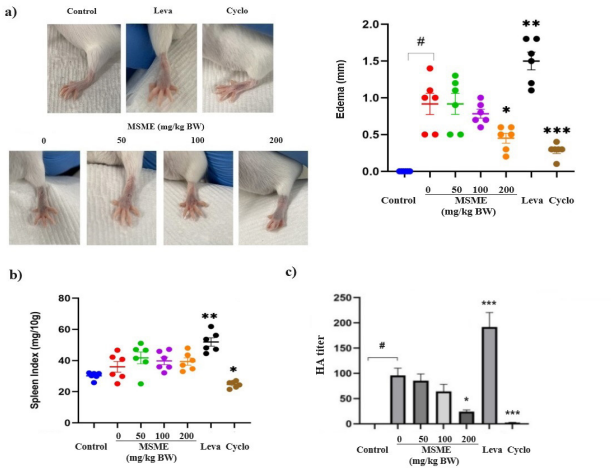

Figure 1: The effect of MSME on DTH mice reaction. All female Balb/c mice, except the control group, were induced with DTH through immunization and challenged with SRBC. (a) The image shows paw edema and the measurement of edema thickness of control and SRBC-induced DTH mice untreated and treated with Mitragyna speciosa methanolic extract (MSME), levamisole (Leva), or cyclophosphamide (Cyclo) are presented in graph mean ± SEM (n=6). The spleen and blood were collected for (b) spleen index and (c) antibody production by hemagglutinin (HA) titer, respectively, and the data is presented as mean ± SEM (n=6). A statistically significant difference (One-way ANOVA) is denoted by #p<0.05 compared to control, while *p<0.05, **p<0.01, and ***p<0.001 indicate a significant difference compared to untreated DTH.

Table I: The effect of MSME on complete blood count of SRBC-induced DTH mice.

Treatment	Dose (mg/kg BW)	RBC ($\times10^{12}$/ml)	Hb (g/L)	WBC ($\times10^{12}$/ml)	Lymphocytes ($\times10^{9}$/L)	Monocytes ($\times10^{9}$/L)
Control	-	6.88 ±0.80	127.3 ±12.35	2.73 ±1.09	2.07 ±0.67	0.36 ±0.25
			SRBC			
MSME	0	7.95 ±0.62	139.3 ±2.40	5.03 ±0.68	3.63 ±0.63	0.56 ±0.31
MSME	50	8.30 ±0.22	124.3 ±10.20	2.77 ±0.47	2.04 ±0.23	0.32 ±0.25
MSME	100	7.02 ±0.89	112.3 ±13.38	3.23 ±0.43	1.82 ±0.24	0.61 ±0.27
MSME	200	6.57 ±1.26	118.7 ±13.32	2.23 ±0.88	1.59 ±0.77	0.36 ±0.13
Leva	2.5	9.92 ±0.40	144.3 ±3.180	8.87 ±1.64[a,b]	7.30 ±1.38[a,b]	1.67 ±0.17[a,b]
Cyclo	30	5.14 ±0.94	87.67 ±4.33[a,b]	1.30 ±0.15[b]	0.73 ±0.26	0.08 ±0.04

MSME: Mitragyna speciosa methanolic extract; SRBC: sheep red blood cells; DTH: delayed-type hypersensitivity; Leva: Levamisol; Cyclo: Cyclophosphamide, RBC: red blood cell, Hb: hemoglobin, WBC: white blood cell
[a] indicates a significant difference (p<0.05) in comparison with control.
[b] indicates a significant difference (p<0.05) in comparison with untreated (0 mg/kg MSME) DTH.

The impact of MSME on splenocyte phenotypes

Figure 2 shows the immunophenotyping analysis of lymphocyte designated CD4 T helper cells (Figure 2a), CD8 cytotoxic T cells (Figure 2b), and CD19 B cells (Figure 2c). The dot plots of each type of lymphocyte population showed no alteration observed to indicate morphological changes (granularity and size) to the cells following stimulation or treatment. In addition, treatment of SRBC-induced DTH mice with MSME only exhibited a marginal increase without any significant difference in the total number of each lymphocyte population compared to the control group (Figure 2d). On the other hand, levamisole displayed a substantial augmentation in the percentage of CD4 (p=0.0041), CD8 (p<0.0001), and CD19 (p=0.0471) populations. In contrast, DTH mice that received cyclophosphamide showed a reduction in the number of CD4 (p=0.0111) and CD19 (p=<0.0001). However, no significant change was observed in the CD8 population.

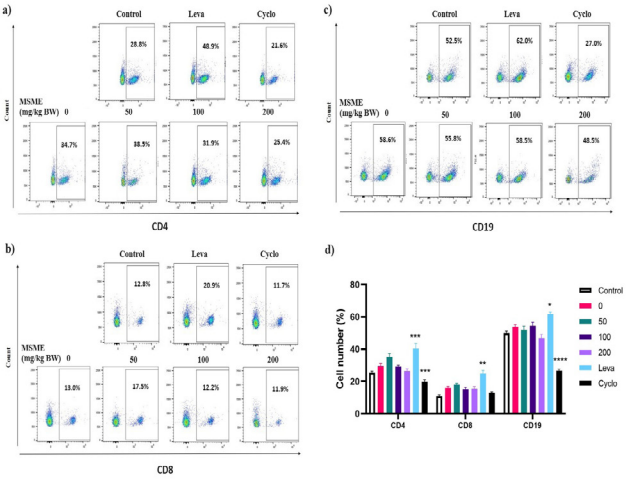

Figure 2: The effect of MSME on lymphocyte phenotypes in SRBC-induced DTH mice. The splenocytes (106 cells/ml) from DTH mice treated with Mitragyna speciosa methanolic extract (MSME), levamisole (Leva), or cyclophosphamide (Cyclo) were stained with fluorochrome-conjugated (a) CD4, (b) CD8, and (c) CD19. (d) The graph shows the average data of CD4, CD8, and CD19 from three independent experiments. Error bars are mean ±SEM. *p<0.05, **p < 0.001, ***p < 0.0001, and ****p < 0.00001 (Two-way ANOVA) indicate a significant difference in comparison with the control group.

MSME inhibits concanavalin A- and lipopolysaccharide-induced lymphocyte proliferation

The splenocytes isolated from SRBC-induced DTH mice were re-stimulated *ex vivo* with LPS or Con A for B or T lymphocyte activation and proliferation, respectively. Figure 3 shows that lymphocytes from the previously treated MSME *in vivo* have no discernible effect in the absence of LPS or Con A. On the other hand, the percentage of lymphocyte proliferation improved in the presence of LPS (from 148.942±6.374 % to 210.899±3.866 %; p<0.0001) or Con A (from 151.273 ±6.111% to 212.509 ±4.876%; p<0.0001). However, the presence of LPS or Con A in MSME-treated cells was reduced in a dose-dependent manner. For example, re-stimulation of MSME-treated splenocytes from DTH mice with LPS reduced the cell proliferation rate by approximately 28.06% (50 mg/kg), 41.26% (100 mg/kg), and 67.22% (200 mg/kg).

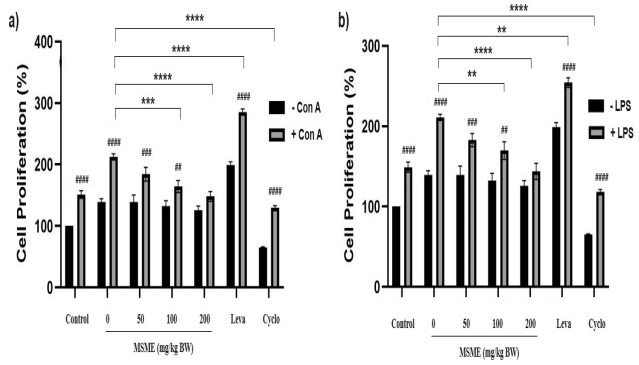

Figure 3: The effect of MSME on splenocyte proliferation upon stimulation with different antigens. Splenocytes from the SRBC-induced DTH mice were isolated and cultured for 72h in the absence or presence of (a) concanavalin A (Con A) or (b) lipopolysaccharide (LPS). The MTT assay was performed to evaluate the cell proliferation rate. The data is represented as mean ± SEM (n=6) and analysed by Two-way ANOVA. ##p<0.001, ####p<0.00001, and ###p<0.0001 indicate a statistically significant difference in comparison with unstimulated control (black bar) within the group. **p<0.001, ***p<0.0001, and ****p<0.00001 indicate a statistically significant difference in comparison with LPS- or Con A-stimulated untreated DTH.

DISCUSSION

M. speciosa is one of the medicinal plants that previous studies have proven to be abundant in potent compounds such as alkaloids, tannins, saponins, flavonoids, phenols, steroids, and carotenoids, affirming its pharmacological attributes, such as anti-inflammatory, antinociceptive, antibacterial, antioxidant, and more [4,6,15,16]. Previously, we have highlighted the potential antioxidative, anti-diabetes [16], and anti-inflammatory properties of MSME in macrophages [4]. Additionally, studies found that intraperitoneal administration of methanol extract from *M. speciosa* (at doses of 100 and 200 mg/kg) effectively and dose-dependently inhibited the development of carrageenan-induced paw edema in rats ($p < 0.05$). A notable decrease in granulomatous tissue formation was also observed [6]. However, no further investigation was performed to evaluate the effects of the extract on the immune response, particularly to the adaptive immune cells. Thus, the present study unveils the immunomodulatory activity of MSME on adaptive cellular and humoral immunity using the DTH animal model.

Levamisole is an immunostimulant drug that was originally used as an anthelmintic. Over time, it has also been recognized for its immunomodulatory properties and used in cancer therapy [17]. However, the use of levamisole in certain disease conditions has been associated with several autoimmune disorders [18]. On the other hand, cyclophosphamide is an immunosuppressant drug that is used to treat various types of cancer and autoimmune diseases [19,20]. However, these synthetic immunomodulatory drugs have potential adverse effects and require proper monitoring [21]. Using levamisole and cyclophosphamide as control drugs will help evaluate the immunomodulatory activity of the target bioactive compound on the immune responses.

Delayed-type hypersensitivity is characterized by a cellular immune response that develops within hours to days and persists for 2-3 days upon exposure to an antigen. It involves antigen recognition by antigen-specific $CD4^+$T cells via antigen-presenting cells (APCs) such as dendritic cells [22,23]. This antigen recognition leads to the release of cytokines and activation of macrophages, resulting in inflammation, heightening vascular permeability, triggers vasodilation, and tissue damage at the site of antigen exposure which subsequently promotes the formation of edema [23].

In the present study, SRBC-, as a source of T-dependent antigen [24,25], induced DTH reaction in a mice inflammatory model was used to evaluate the immunomodulatory effects of MSME on the adaptive immune response. Immunizing and challenging the animals with SRBC triggers T cell-mediated DTH reaction and induces the development of edema. The formation of paw edema increased with the SRBC challenge. Following oral administration of MSME, a significant reduction of paw edema was observed in a dose-dependent manner. In addition, the production of anti-SRBC antibodies that increased during DTH reaction was inhibited by MSME. The augmentation of antibody production to SRBC reveals the increased responsiveness of T-dependent B cell-mediated humoral immunity and it was later inhibited by MSME. The attenuation of paw edema formation and reduction of anti-SRBC antibodies in SRBC-induced DTH mice suggests the suppressive activity of MSME against T and B cell-mediated immune response.

To further elucidate the effects of MSME on immune cell functions, the spleen weight, CBC, and lymphocyte subset population in SRBC-induced DTH mice were analyzed. Results show no significant alteration has been attributed to MSME on spleen weight, CBC, and lymphocyte subset population. These findings suggest that MSME merely affects cellular activity without interfering with cell development in the bone marrow. Previously, the investigation of the sub-chronic toxicity of standardized methanolic extract of *M. speciosa* revealed that oral administration of the extract (at doses of 100, 200, and 500 mg/kg) in Sprague-Dawley rats did not cause alterations in various blood parameters including RBC, WBC, hemoglobin, hematocrit, and platelet [26]. Similarly, the acute toxicity of the standardized methanolic M. speciosa extract (at doses of 100, 500, and 1000 mg/kg BW) revealed no discernible impact on hematological parameters and organ index, including those of the heart, liver, lungs, spleen, kidney, and brain [26]. Furthermore, sub-chronic exposure of rats to mitragynine showed no significant effect on organ weights, with no notable changes observed in the weights of the same organs [27]. Interestingly, long-term

consumption of M. speciosa does not affect lymphocyte, neutrophil, and monocyte counts in thirteen regular users [28].

Excessive production of cytokines, coupled with hyperproliferation of T cells, precipitates autoimmune reactions leading to tissue damage in autoimmune diseases such as multiple sclerosis. Thus, regulating $CD4^+$ and $CD8^+$ T cell function is crucial in preventing autoimmune diseases [29,30]. In the present study, the proliferation of lymphocytes isolated from mice previously induced with SRBC and received MSME treatment was further evaluated in response to Con A or LPS stimulation *ex vivo*. Stimulation of lymphocytes with Con A or LPS increased the cell proliferation rate in SRBC group and gradually decreased in MSME-treated groups. This finding demonstrates the anti-proliferative activity of MSME regardless of antigenic stimulation. Our data also suggest that MSME could potentially reduce chronic inflammatory conditions through attenuation of inflammatory response and cell proliferation, without compromising the cell morphology and phenotype.

Building on the findings of this study, several areas of future research warrant attention. Firstly, further investigation is needed to explore the effects of MSME on other disease models such as cancer or infection. Moreover, employing another method such as ELISA to quantify the level of antibody production could enhance data accuracy. Finally, further investigation into the molecular mechanisms and cellular signaling could enrich our understanding of the immunomodulatory activity of MSME.

CONCLUSION

Drawing from the aforementioned data, we propose that MSME exhibits immunomodulatory activity through suppression of SRBC-induced cellular and humoral immune responses and antiproliferative activity in an *in vivo* DTH animal model without affecting the homeostatic activity of the immune cells. These findings demonstrate the potential of *M. speciosa* to be further investigated and developed as an immunotherapeutic agent.

ACKNOWLEDGEMENT

This study was funded by the Graduate Putra Grant (GP-IPS) (grant number 9726100) from Universiti Putra Malaysia.

REFERENCES

1. Ganeshpurkar A, Saluja AK. Experimental animal models used for evaluation of potential immunomodulators: A mini review. *Bull Fac Pharmacy, Cairo Univ.* 2017;55(2):211-216. doi:10.1016/j.bfopcu.2017.08.002
2. Avery BA, Boddu SP, Sharma A, et al. Comparative Pharmacokinetics of Mitragynine after Oral Administration of Mitragyna speciosa (Kratom) Leaf Extracts in Rats. *Planta Med.* 2019;85(4):340-346. doi:10.1055/a-0770-3683
3. Karunakaran T, Ngew KZ, Zailan AAD, Mian Jong VY, Abu Bakar MH. The Chemical and Pharmacological Properties of Mitragynine and Its Diastereomers: An Insight Review. *Front Pharmacol.* 2022;13(February):1-11. doi:10.3389/fphar.2022.805986
4. Kafo ASK, Elsalami RM, Zailan NFZ, et al. Effects of Mitragyna Speciosa (Korth .) on macrophage immune responses. *Curr Trends Biotechnol Pharm.* 2023;17(October):121-130.
5. Tohar N, Shilpi JA, Sivasothy Y, Ahmad S, Awang K. Chemical constituents and nitric oxide inhibitory activity of supercritical carbon dioxide extracts from Mitragyna speciosa leaves. *Arab J Chem.* 2016;12(3):350-359. doi:10.1016/j.arabjc.2016.09.005
6. Mossadeq SWM, Sulaiman MR, Tengku Mohamad TA, et al. Anti-inflammatory and antinociceptive effects of Mitragyna speciosa Korth methanolic extract. *Med Princ Pract.* 2009;18(5):378-384. doi:10.1159/000226292
7. Yuandani, Nugraha S, Laila L, Satria D. Immunomodulatory effects of standardized extract of Curcuma mangga val. On cytokines, antibody and delayed-type hypersensitivity response in Wistar rats. *Res Pharm Sci.* 2021;16(1):16-25. doi:10.4103/1735-5362.305185
8. Hamid K, Sah S, Kalgo M, et al. Immunostimulatory Activity of Aqueous Extract of Polyherbal Formulation on Th1/Th2 Cytokines Secretion and Cell Mediated Immune Response in Rats. *Saudi J Med Pharm Sci.* 2021;7(1):64-70. doi:10.36348/sjmps.2021.v07i01.013
9. Kumolosasi E, Ibrahim SNA, Shukri SMA, Ahmad W. Immunostimulant activity of standardised extracts of mangifera indica leaf and curcuma domestica rhizome in mice. *Trop J Pharm Res.* 2018;17(1):77-84. doi:10.4314/tjpr.v17i1.12
10. Ahmad W, Jantan I, Haque MA, Arsyad L. Magnoflorine from Tinospora crispa upregulates innate and adaptive immune responses in Balb/c mice. *Int Immunopharmacol.* 2022;111(July):109081. doi:10.1016/j.intimp.2022.109081
11. Mao R, Wu L, Zhu N, et al. Immunomodulatory effects of walnut (Juglans regia L.) oligopeptides on innate and adaptive immune responses in mice. *J Funct Foods.* 2020;73(February):104068. doi:10.1016/j.jff.2020.104068
12. Kumar S, Sharma G, Sidiq T, et al. Immunomodulatory potential of a bioactive fraction from the leaves of Phyllostachys bambusoides (bamboo) in BALB/c mice. *EXCLI J.* 2014;13:137-150.
13. Yun L, Wu T, Li Q, Zhang M. Dietary

supplementation with purified wheat germ glycoprotein improve immunostimulatory activity in cyclophosphamide induced Balb/c mice. *Int J Biol Macromol.* 2018;118:1267-1275. doi:10.1016/j.ijbiomac.2018.06.199
14. Nga NTH, Ngoc TTB, Trinh NTM, Thuoc TL, Thao DTP. Optimization and application of MTT assay in determining density of suspension cells. *Anal Biochem.* 2020;610(April):113937. doi:10.1016/j.ab.2020.113937
15. Sabetghadam A, Ramanathan S, Mansor SM. The evaluation of antinociceptive activity of alkaloid, methanolic, and aqueous extracts of Malaysian Mitragyna speciosa Korth leaves in rats. *Pharmacognosy Res.* 2010;2(3):181-185. doi:10.4103/0974-8490.65514
16. Zailan NF, Seri Narti S, Hassan M. Evaluation of Phytochemical Composition , Antioxidant and anti- Diabetic Activities of Mitragyna speciosa Methanolic Extract (MSME). *Malaysian J Med Heal Sci*. 2022;18(3):92-99. doi:10.47836/mjmhs18.s21.15
17. Stevenson BHC, Green I, Hamilton JM, Calabro BA, Parkinson DR. Results , and Future Applications to the Treatment of Cancer. 2012;9(11):2052-2066.
18. AM McGrath AM. A Comparison of Intra-Articular Hyaluronic Acid Competitors in the Treatment of Mild to Moderate Knee Osteoarthritis. *J Arthritis.* 2013;02(01):1-5. doi:10.4172/2167-7921.1000108
19. Ahlmann M, Hempel G. The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy. *Cancer Chemother Pharmacol.* 2016;78(4):661-671. doi:10.1007/s00280-016-3152-1
20. Horimoto AMC, Matos ENN, da Costa MR, et al. Incidence and prevalence of systemic sclerosis in campo grande, state of Mato Grosso do Sul, Brazil. *Rev Bras Reumatol.* 2017;57(2):107-114. doi:10.1016/j.rbre.2016.09.005
21. Bascones-Martinez A, Mattila R, Gomez-Font R, Meurman JH. Immunomodulatory drugs: Oral and systemic adverse effects. *Med Oral Patol Oral Cir Bucal.* 2014;19(1). doi:10.4317/medoral.19087
22. Uzzaman A, Cho SH. Classification of hypersensitivity reactions. *Allergy Asthma Proc.* 2012;33(SUPPL. 1):96-99. doi:10.2500/aap.2012.33.3561
23. Basu S, Banik BK. Hypersensitivity : An Overview. *Immunol Curr Res.* 2018;2(1):2-3.
24. Stamm C, Barthelmann J, Kunz N, Toellner KM, Westermann J, Kalies K. Dose-Dependent Induction of Murine Th1/Th2 Responses to Sheep Red Blood Cells Occurs in Two Steps: Antigen Presentation during Second Encounter Is Decisive. *PLoS One.* 2013;8(6). doi:10.1371/journal.pone.0067746
25. Bergström JJE, Heyman B. Mice immunized with IgG anti-sheep red blood cells (SRBC) together With SRBC have a suppressed Anti-SRBC antibody response but generate germinal centers and anti-IgG antibodies in response to the passively administered IgG. *Front Immunol.* 2017;8(AUG). doi:10.3389/fimmu.2017.00911
26. Ilmie MU, Jaafar H, Mansor SM, Abdullah JM. Subchronic toxicity study of standardized methanolic extract of mitragyna speciosa korth in sprague-dawley rats. *Front Neurosci.* 2015;9(MAY):1-6. doi:10.3389/fnins.2015.00189
27. Sabetghadam A, Ramanathan S, Sasidharan S, Mahsufi S. Subchronic exposure to mitragynine , the principal alkaloid of Mitragyna speciosa , in rats. *J Ethnopharmacol.* 2013;146(3):815-823. doi:10.1016/j.jep.2013.02.008
28. Singh D, Narayanan S, Grundmann O, et al. Long-term effects of kratom (mitragyna speciosa) use. *Malaysian J Med Heal Sci.* 2020;16(4):64-72.
29. Endo K, Sawa T, Tanaka Y, et al. Oral administration of procyanidin B2 3,3″-di-O-gallate ameliorates experimental autoimmune encephalomyelitis through immunosuppressive effects on CD4+ T cells by regulating glycolysis. *Eur J Pharmacol.* 2023;954(February):175879. doi:10.1016/j.ejphar.2023.175879
30. Yang X, Jiang Y, Yang J, et al. Prenylated flavonoids, promising nutraceuticals with impressive biological activities. *Trends Food Sci Technol.* 2015;44(1):93-104. doi:10.1016/j.tifs.2015.03.007