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Modulation Effect of *Muntingia calabura* Leaves Extracts Supplementation in High Fat Diet-Administrated BALB/c Mouse

Wira Eka Putra[1], Intan Nilatus Shofiyah[2], Adelia Riezka Rahim[2], Arief Hidayatullah[3], Muhaimin Rifa'i[2,*]

[1] *Biotechnology Study Program, Department of Applied Sciences, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, East Java, Indonesia.*

[2] *Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, East Java, Indonesia*

[3] *United Nations Development Programme Indonesia, Health Governance Initiative, Eijkman-RSCM Building, Jakarta, Indonesia*

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Abstract

Hyperglycemia is recognized as the primary indicator of diabetes. The occurrence of Hyperglycemia results from a combination of contributing factors, such as dietary habits, exercise routines, and overall lifestyle choices. Accumulating evidence showed the hyperglycemia also causes inflammation, which changes the body's cellular and immune systems. Similarly, the acute hyperglycemia increases the levels of pro-inflammatory cytokines such as TNF-α and IL-6. The present study aimed to determine the effect of *Muntingia calabura* leaves extracts administration toward the immune system in hyperglycemia-induced high-fat diet in mouse model. Five experimental groups were applied in this study, including the control group, hyperglycemic group, and three groups treated with *M. calabura* leaves extracts in several doses such as 400, 700, and 2800 mg/kg BW. Flow cytometry analysis was performed to evaluate the inflammation markers such as IL-1β, IFN-ɣ, or TNF-α on the subset of macrophages and Th1 cells. Our findings indicate that the extracts did not enhance or elevate the production of IL-1β by the macrophage subgroup/population. Similarly, the pro-inflammatory cytokines such as IFN-ɣ and TNF-α were reduced on the CD4 after treated with the extracts. Therefore, this study demonstrated that *M. calabura* leaves extracts exerts suppressive activity to maintain the physiological level of immune system. To be highlighted, the immunosuppression action reduced several inflammatory markers, including macrophages and Th1 cells. Thus, the present study suggested that *M. calabura* leaves extracts possibly become alternative and complementary medicine against hyperglycemia.

Keywords: Hyperglycemia, inflammation, plant, macrophages, Th1 cells

Introduction

People's lifestyles have been greatly affected by the rising trend of unhealthy eating habits. Diets high in calories, saturated fats, salt, and added sugars, combined with a sedentary lifestyle, can lead to obesity, insulin resistance, and hyperglycemia [1-3]. Insulin resistance is linked to metabolic syndrome, specifically type II diabetes mellitus (T2DM), and is caused by irresponsive insulin receptors, decreased expression of glucose transporters, and significantly reduced glucose intake by cells. Together these factors contribute to the development of hyperglycemia [4, 5]. Unfortunately, hyperglycemia affects around 462

million people globally, or roughly 6% of the global population, and was responsible for 1 million deaths in 2017 alone [6].

Hyperglycemia has been identified as the primary symptom of diabetes. It also produces inflammation, which alters the cellular and immune systems of the body. Diabetic patients also have an inadequate immune response that fails to control the spread of invading microorganisms [7]. Acute hyperglycemia may increase the levels of pro-inflammatory cytokines such as TNF-α and interleukin-6 (IL-6) which become main indicator of inflammation, with hyperglycemia patients demonstrating an increase in TNF-α [8, 9]. TNF-α is a major adipokine generated by adipocytes that influences insulin sensitivity, especially in response to chronic inflammation induced by obesity [10, 11]. Adipocytes also emit numerous adipokines during these conditions, including IL-6, IL-1β, and monocyte chemoattractant protein 1 (MCP-1) [12]. The pro-inflammatory cytokine IL-1β controls lipid metabolism, insulin levels, and lipase activity. Because active IL-1β suppresses the insulin signalling pathway, it may be a measure of insulin resistance [13].

The underlying problem with diabetes is that there is no diabetic cure, despite the fact that it can be treated and controlled, and some people can go into remission [14]. The increasing prevalence of diabetes in recent times has led to a growing need for blood sugar management treatments. This includes both synthetic drugs and natural remedies designed to combat high blood glucose levels. As a result, the market for these anti-diabetic therapies has expanded significantly. Traditional herbal medicines are thought to have anti-diabetic qualities as well as the potential to relieve its symptoms [15]. In the case of diabetes, however, the boundary between effective treatments is hazy. Glycemia, one of the primary variables, must presumably be improved or normalized as part of the remission requirements [16].
Muntingia calabura is a plant that is commonly utilized in traditional medicine. It is often known as the Jamaican Cherry [17]. South Asian and South American communities have extensively used this plant in traditional medicine to relieve headaches and prostate problems and reduce gastric ulcers [18, 19]. In Indonesia, the local community commonly consumes the fruit, while the leaves are brewed to cure fever and muscular spasms and as an antiseptic. The *M. calabura's* leaves extract contains a high number of flavonoids, flavone, and steroidal compounds that could act as anti-diabetic and anti-inflammatory agents [17]. Accumulating evidence showed that *M. calabura* contains various bioactive components including geniposide, luteolin, daidzein, quercetin, kaempferol, formononetin, 6-hydroxyflavone, gallic acid, kaempferide, genistein, and chrysin [20, 21]. There have been several research studies on the effects of *M. calabura* leaves extracts, but very few discuss its impacts on the immune system during hyperglycemia. Pharmacological research revealed anticancer, antinociceptive, anti-inflammatory, antipyretic, antibacterial, anti-diabetic, and antistaphylococcal properties [17]. Thus, the present study aimed to investigate the immunomodulatory effect of *M. calabura* leaves extracts in high-fat diet-induced hyperglycemia mouse model.

Materials and Methods

Muntingia calabura leaves extraction procedure

M. calabura leaves extractions was implemented by applying the decoction with freeze drying technique. The Materia Medika Batu supplied the dried *M. calabura* leaves simplicia. The simplicia was then boiled in distilled water for 2 hours at 80ºC in a 1:10 (w/v) ratio. The mixture was then filtered twice using grade 1 Whatmann filter paper. The filtrate was frozen at -70°C in a freezer for 24-48 hours. The filtrate then freeze-dried for at least 24 hours or until no solvent remains.

Hyperglycemia induction

Hyperglycemia was induced by administrating a high-fat diet (HFD) for 12 weeks in BALB/c mice. The HFD was created with modifications based on Saravanan and Pari [1]. The following ingredients were used: 80 g duck egg yolk, 170 g beef fat, 300 g liquid fructose, 350 g HiGro 551, and 100 g high protein wheat flour. All ingredients are mixed until homogeneous, then shaped into biscuits on a baking sheet, baked at 150ºC for 10 minutes, repeated, and kept at room temperature. Drinking water is provided by adding liquid fructose to up to 20% of the total amount of the water supplied.

Oral administration of the M. calabura leaves *extracts*

Each mouse was weighed separately for certain dosage treatment, and then the *M. calabura* leaves extracts were then quantified based on the average body weight of the mice and diluted in distilled water to the prescribed volume of solution. The treatment group received the specified amount of medication in accordance with the recommended dosage protocol. For two weeks, *M. calabura* leaves extract was administered every day orally. The extract was not administered to control or the three of hyperglycemic groups.

Animal model description and experimental design

A total of 25 male BALB/c mice aged three weeks were used in this study. These pathogen-free mice were purchased from Gadjah Mada University, Yogyakarta. They were housed in a designated area for experimental animals at room temperature and fed regularly. The study employed a completely randomized design with five treatment groups and five replications. The mice were divided into the following groups: control group, hyperglycemic group (12 weeks of HFD), HD1 (12 weeks of HFD + 2 weeks of *M. calabura* leaves extract with 420 mg/kg BW), HD2 (12 weeks of HFD + 2 weeks of *M. calabura* leaves extract with 700 mg/kg BW), and HD3 (12 weeks of HFD + 2 weeks of *M. calabura* leaves extract with 2800 mg/kg BW dosage). This study has been passed the ethical clearance in handling and care of experimental animals with certificate No 670-KEP-UB issued by The Research Ethics Committee of Brawijaya University, Malang.

Splenocytes isolation

At the conclusion of the treatment period, all experimental mice were euthanized, dissected, and their splenocytes were extracted from the spleen. The spleen was washed in PBS before being transferred to sterile PBS and crushed with the bottom tip of the syringe by squeezing and spinning counter clockwise. PBS was added to the solution in a propylene tube until the volume reached 6 ml. The suspension was then centrifuged for 5 minutes at a speed of 2500 rpm at a temperature of 10ºC. The pellets obtained were suspended with 1 mL of sterile PBS.

Flow cytometry preparation and analysis

The obtained pellet suspension was put in a microtube containing 500 µl of PBS and centrifuged at 2500 rpm for 5 minutes at a temperature of 10ºC. Extracellular staining was performed by adding 50 µl of certain extracellular antibody such as FITC-conjugated rat-anti mouse CD4 or FITC-conjugated rat-anti mouse CD68 to the pellet and incubating it in the dark for 20 minutes at 4ºC. Intracellular staining was performed following extracellular staining. For intracellular staining, the suspension was pipetted and treated for 20 minutes at 4ºC with cytofix-cytoferm. Then 500 µl of washperm was added and centrifuged for 5 minutes at 2500 rpm and 10ºC. PE-conjugated rat-anti mouse TNF-α, PE/Cy5-conjugated rat anti-mouse IFNγ, or PE/Cy5- conjugated rat anti-mouse IL-1β PE/Cy5- conjugated rat anti-mouse IL-1β were add to the pellet and incubate in the dark for 20 minutes at 4ºC. It was then

resuspended in 300 µl of PBS, placed in a cuvette, and processed through the FACS Calibur™ (BD Biosciences) flow cytometry.

Statistical analysis

The flow cytometry data were processed with CellQuest™ software (BD Biosciences) and statistically assessed using SPSS (v.16) program. The data were examined using one-way ANOVA followed by a Tukey's HSD post-hoc test. If p value < 0.05, there was a significant difference between treatments.

Results and Discussion

The effect of M. calabura leaves *extracts on CD68$^+$ macrophages expression*

In the present study, the role of *M. calabura* leaves extracts was evaluated toward several inflammation markers, such as the expression of macrophages and Th1 cells (Figure 1). Macrophages, also known as CD68$^+$ cells, play several important roles in the body's defence system. These cells release signaling proteins called cytokines, which trigger and control inflammatory responses. They also break down and eliminate pathogens, while promoting tissue repair and regeneration. Pathogens or cell damage may activate macrophage defense mechanisms, leading them to launch the innate immune response by binding to Toll-Like Receptors (TLRs) and NOD-Like Receptors (NLRs) [13, 22]. The number of CD68$^+$ macrophage cells in hyperglycemic model mice was significantly greater than in healthy mice (Figure 2). This finding was consistent with another study that found hyperglycemia might increase the number of macrophages in several organs, including the spleen. The increased number of macrophages is assumed to be the consequence of hyperglycemia-induced proliferation in situ, notably in the spleen [23], in response to hyperglycemic upregulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) [24]. A high quantity of GM-CSF will bind to their respective receptors, phosphorylate JAK2, and then activate the MAPK/ERK pathway, causing macrophage growth [25, 26]. Since CD68 expression is a macrophage marker, this evidence suggests that GM-CSF expression correlates with macrophage number [27].

Furthermore, hyperglycemia has two main effects: elevated CD68 expression and oxidative stress [28, 29]. The relationship between these two items is yet unclear. However, it is hypothesized that oxidative stress is one of the primary drivers of increased CD68 expression since oxidative stress induced by reactive oxygen species (ROS) is anticipated to activate NF-κB pathway, either canonically or noncanonically [30]. On the other hand, hyperglycemia alters the phenotype and function of macrophages, causing them to differentiate into a pro-inflammatory phenotype (M1), increasing their ability to endocytose, activate T cells, and generate IL-12 [31–33]. The increase in macrophages was inversely associated with the number of pancreatic cells producing insulin. The higher the number of macrophages, the higher the production of IL-1β, which induces the pancreatic beta-cell to undergo apoptosis and reduces insulin secretion capacity [34, 35].

Specifically, compared to the hyperglycemic control group, two weeks of administration of all dose of *M. calabura* leaves extract resulted in a reduction in the number of CD68$^+$ cells (Figure 2). As previously stated, *M. calabura* extract has a high concentration of flavonoids, flavones, and steroidal/triterpenoid components. Because they reduce oxidative stress formation through suppression of NF-κB, inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS) pathways, genistein and kaempferol in the extract are hypothesized to decrease CD68 expression and macrophage proliferation [36, 37]. It also reduces GM-CSF expression and responsiveness in macrophages, reducing their proliferation [38]. Gallic acid and daidzein are considered to decrease the production of monocyte chemoattractant protein-1 (MCP-1) by inflamed tissue such as fat, decreasing macrophage recruitment to the site of inflammation and, as a result, macrophage proliferation [39, 40].

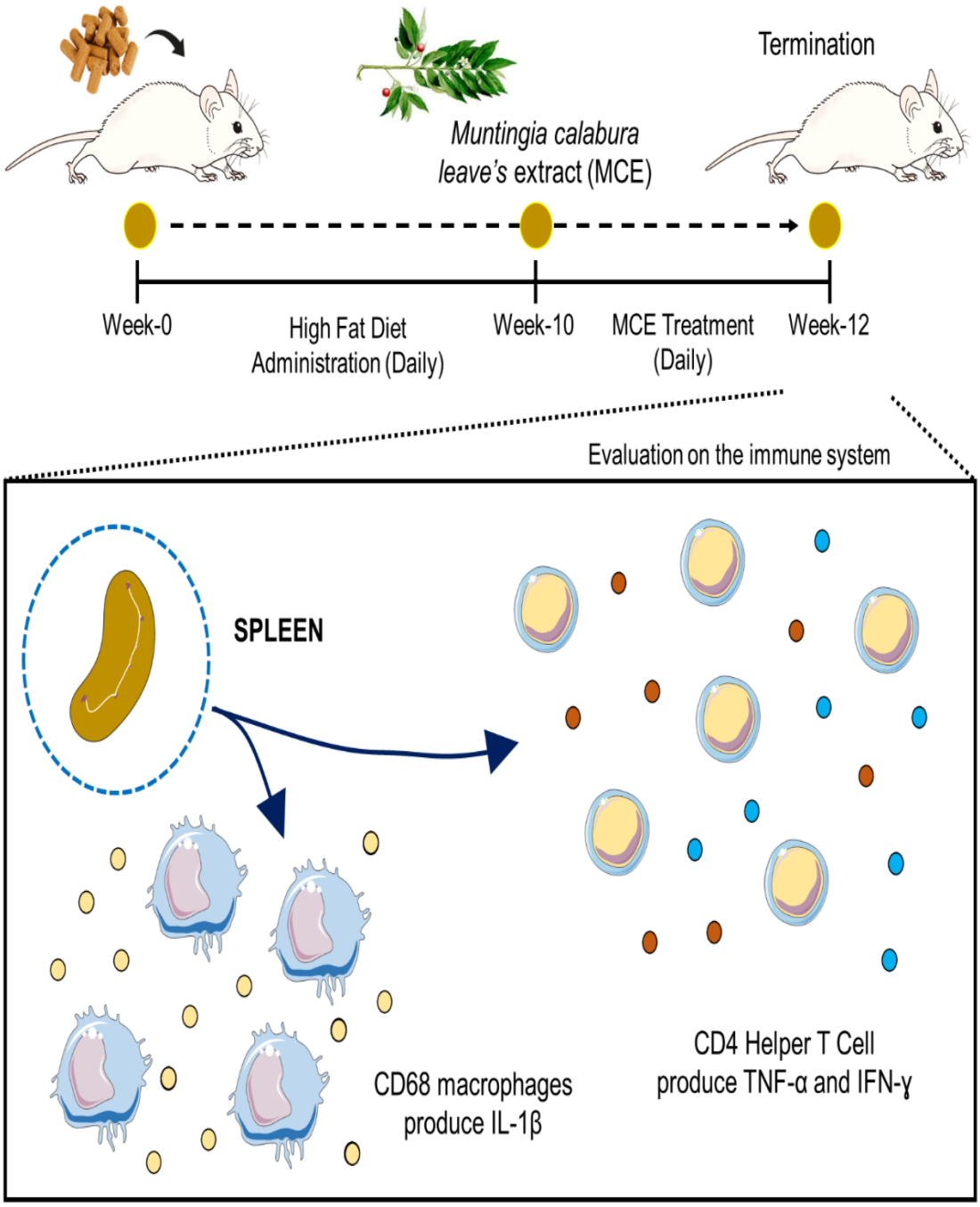

Figure 1: Schematic image showed modulation effect of *M. calabura* leaves extracts toward macrophages and Th1 cells in high fat diet-administrated mouse into normal condition.

The high concentration of flavonoids, flavones, and steroidal/triterpenoid compounds in *M. calabura* leaves extracts was thought to be the main cause of the reduction in $CD68^+$ cells due to the presence of the compounds inhibited the proliferation of $CD68^+$ cells significantly by inhibiting CSF synthesis and responses. CSF-1 begins the mitogenic response by binding to the CSF-1 receptor, which activates receptor tyrosine kinases and initiates mitogenic signals via the effector route such as Erk1/2, Akt and STAT3 pathways [41]. Flavonoids can serve as antiproliferation agents of $CD68^+$ cells by inhibiting tyrosine kinase's work, suppressing the mitogenic activity of $CD68^+$ cells. Although flavonoids could block CSF-1

activity and therefore reduce proliferation, they did not affect the viability of $CD68^+$ cells [42].

The effect of M. calabura leaves *extracts on IL-1β$^+$ expressing CD68$^+$ macrophages*

IL-1β is a pro-inflammatory cytokine that promotes inflammation and primarily acts as an inflammatory mediator in response to infection or other stimuli, synthesized primarily by hematopoietic cells [43, 44]. Normally, IL-1β plays a crucial role in homeostatic activities such as body temperature and appetite control and is involved in various cellular activities, including cell proliferation, differentiation, and apoptosis. Excessive IL-1β synthesis will result in pathophysiological alterations over the course of the disease [45]. Although macrophages are the primary source of IL-1β, they are also synthesized by epithelial, lymphoid, and vascular organs, including α-pancreatic cells [46].

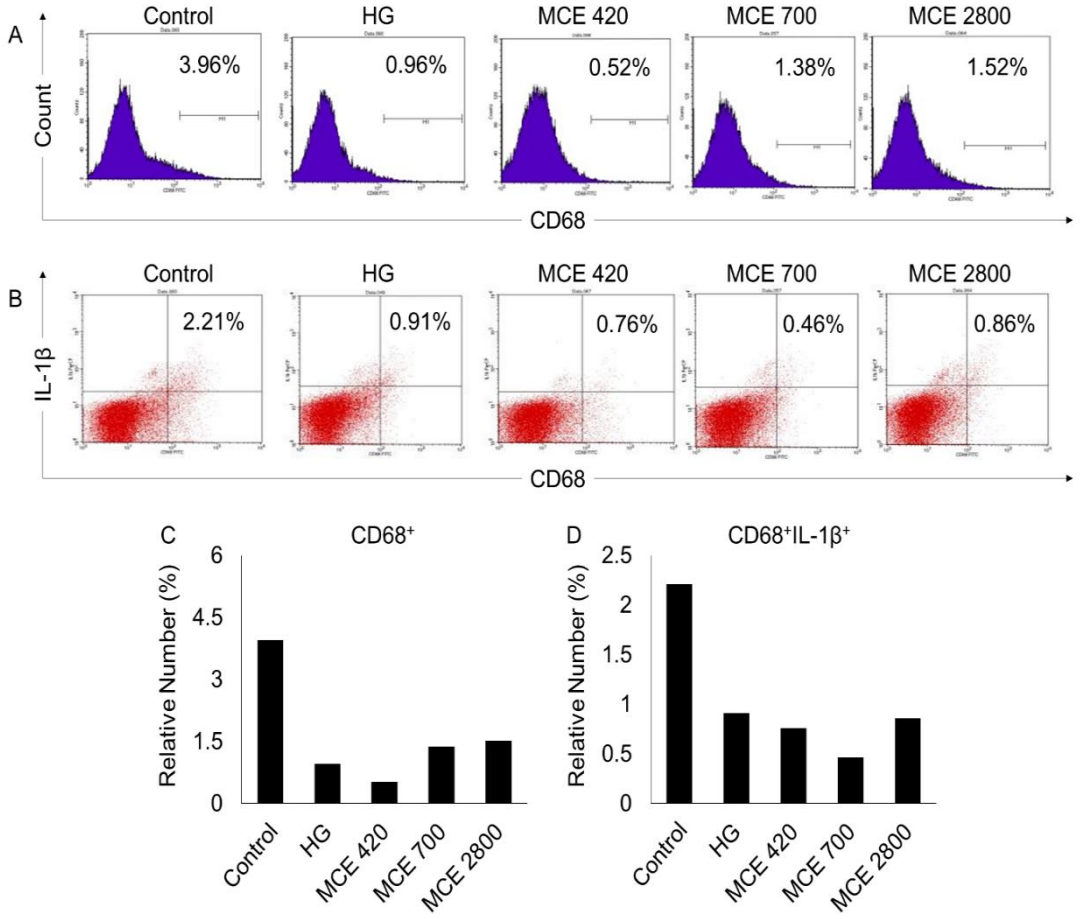

Figure 2: Modulation effects of *M. calabura* leaves extracts toward $CD68^+$ macrophages (A and C) and IL-1β expressing $CD68^+$ macrophages (B and D) in high fat diet-administrated mouse into normal condition.

Hyperglycemic mice boosted the expression of various genes, including those in adipose tissue and subendothelial areas, as well as insulin resistance [45]. Caloric excess during HFD administration causes adipocyte hypertrophy and, as a result, persistent inflammation. This situation may boost the manufacture of pro-inflammatory cytokines, notably TNF-α, leading to the migration of macrophages to adipose tissue [47]. TNF-α is a strong polarizing agent of M1 macrophages via an independent STAT1/IRF-1 pathway [48]. As indicated in the preceding paragraph, hyperglycemia may also activate GM-CSF upregulation pathway. It is

an important growth factor that tends to polarize macrophages towards M1-phenotype, which exhibits a higher degree of pro-inflammatory characteristics by highly secreting pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [31,49], triggered by high-dose induction of GM-CSF [25], which then triggers phosphorylation of GM-CSFR β-chain resulting in JAK/STAT5 cascade activation [49], subsequently activating NF-κB and IRF-5 cascade [26, 50, 51]. The combination of high TNF-α and GM-CSF creates a positive feedback loop, boosting the number of M1 macrophages secreting IL-1β [52].

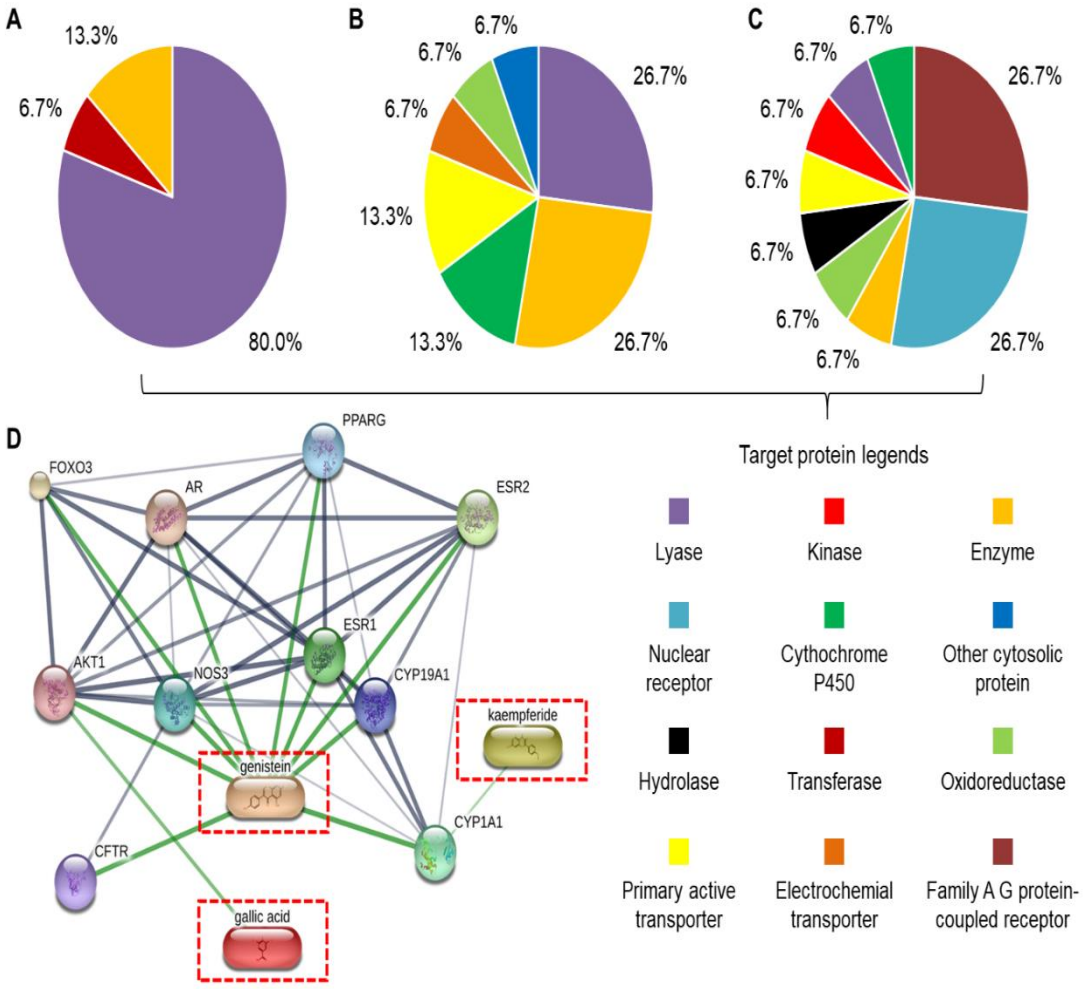

Figure 3: Prediction of *M. calabura* leaves bioactive compounds on target proteins of gallic acid (A), kaempferide (B), genistein (C), and protein interaction network analysis (D) according to the SwissTargetProtein (http://www.swisstargetprediction.ch/) and STITCH (http://stitch.embl.de/) database.

Hyperglycemia also influences IL-1β expression by increasing ROS generation and modifying NF-κB activation to generate IL-1β in macrophages [44]. Hyperglycemia is likely to activate macrophages by blocking AMP-activated protein kinase (AMPK), activating PI3K/AKT signalling pathway, and producing mitochondrial dysfunction, resulting in elevated ROS generation and IL-1β secretion [53]. Because pancreatic β-cells generate a considerably larger quantity of IL-1R, they are more vulnerable to IL-1β-induced death via stimulation of MAPK and NF-κB signalling pathways, reducing the relative proportion of pancreatic cells that express insulin [54].

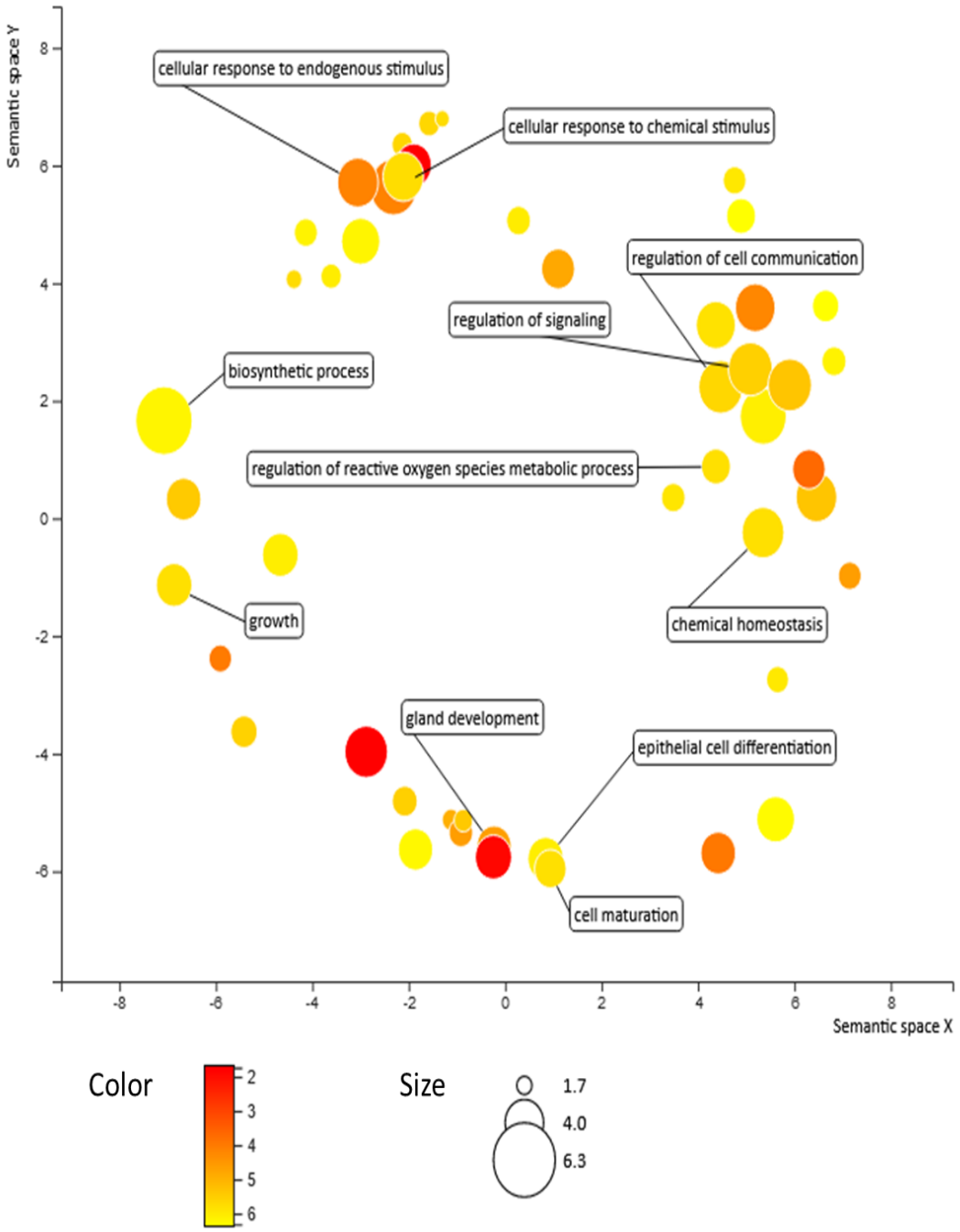

Figure 4: Map visualization of *M. calabura* leaves bioactive compounds biological response. The map showed multiple biological processes were affected mainly by gallic acid, kaempferide, and genistein.

The administration of M. calabura leaf extracts to the hyperglycemic mouse model led to a considerable decrease in IL-1β cytokine production by M1 macrophages compared to untreated hyperglycemic control mice. *M. calabura* leaves extracts administration at dosages of 420 mg/kg BW and 700 mg/kg BW reduced IL-1β expression even lower than in healthy control mice. In hyperglycemic mice, all dosages of *M. calabura* leaves extracts significantly

decreased the production of IL-1β cytokines by M1 macrophages. The low number of IL-1β expression in macrophages might demonstrated that the mouse model underwent the healing process which need anti-inflammatory reaction. Additionally, to validate more information about the biological activity of *M. calabura* leaves bioactive compounds, we provided the predictive results of main compounds of *M. calabura* leaves on protein interaction network and the target protein (Figure 3). More specific, we found that the three of *M. calabura* leaves bioactive compounds namely gallic acid, kaempferide, and genistein have involved in various biological process (Figure 4). Lastly, according to absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis, we found that those compounds are greatly possible to develop into drug candidate (Figure 5).

The *M. calabura* leaves extracts contains flavonoids, flavones, and triterpenoids compounds which act as anti-diabetic and anti-inflammatory agents [17]. Quercetin, for example, is projected to reduce the production of functional IL-1β by decreasing ROS/NF-κB/NLRP3 inflammasome pathway and increasing the expression of IL-10, hence inhibiting NLRP3 inflammasome activation and inflammatory factor secretion [55]. Although this mechanism is yet unclear, kaempferol in the extract is considered to reduce IL-1 expression via blocking ROS/JNK/NF-κB pathways by serving as a phosphorylation inhibitor of NF-κB, which is required for the transcription process of IL-1β gene [56,57]. Genistein and gallic acid are also thought to inhibit IL-1β expression by inhibiting NF-κB and MAPK pathways, but with a less detailed mechanism, except for gallic acid, which is believed to inhibit IκBs degradation [58, 59].

The effect of M. calabura leaves *extracts on IFN-γ$^+$ expressing CD4$^+$ Th1 cells*

The findings revealed that IFN-γ cytokine produced by the CD4+ T cell population, also referred to as Th1 cells, raised noticeably more when hyperglycemia mice were compared to normal mice (Figure 6A and 6C). It is an essential marker for activated Th1 cells. In hyperglycemic mice, increased IFN-γ synthesis in Th1 cells was due to stimulation of IFN-γ secreted by other immune cells such as NK cells. The IFN-γ then binds to IFN-γR on the surface of CD4$^+$ cell, resulting in activation of the receptor-associated tyrosine kinases JAK1/JAK2, leading to the tyrosine phosphorylation and activation of the transcription factor STAT1 homodimers, promoting its translocation to the nucleus, and upregulate the IFN-γ expression [60, 61]. Activated STAT1 and upregulated IFN-γ expression also interfere with the IL-4 expression by inhibiting STAT6 signalling pathway, promoting CD4$^+$ polarization toward Th1 phenotype [62].

Furthermore, hyperglycemia increases the rate of ROS generation in $CD4^+$ cells. Hyperglycemia promotes the generation of superoxide, a component of ROS, by various processes, including the glycolytic pathway and the Krebs cycle. These pathways release NADH and succinate, both of which contribute to the generation of superoxide. Through redox-dependent signalling pathways, superoxide can increase the release of pro-inflammatory cytokines [63]. High glucose levels may cause macrophages to release IL-12, which stimulates $CD4^+$ T cells to produce IFN-γ [64]. Hyperglycemia also can stimulate inflammatory cytokines by stimulating NF-κB through ROS-dependent pathways [63].
Moreover, diabetes mellitus could increase advanced glycation end (AGE) products and AGE-modified proteins, which can bind to AGE receptors on macrophages and T cells, increasing the synthesis of pro-inflammatory cytokines. T cells can secrete an excessive amount of IFN-γ, causing inflammation and oxidative stress in renal tissue. The AGE products also stimulate IFN-γ production, promoting inflammation by activating macrophages and vascular cells. Furthermore, fatty acids derived from the HFD are also thought to promote the release of IFN-γ cytokines by T cells. The IFN-γ plays an important role in the development of hyperglycemia. It plays a direct role in pancreatic β-cell dysfunction. In beta cells, iNOS produces NO, which IFN-γ stimulates in tandem with IL-1β [65]. These cytokines block glucose-stimulated insulin by increasing iNOS level. The IFN-γ also promotes MHC class I expression in pancreatic beta cells, which activates $CD8^+$ T cells, leading to apoptosis [66].

Category	Model Name	Gallic acid	Kaempferide	Genistein	Unit
Absorption	Water solubility	-2.56	-3.238	-3.595	log mol/L
	Caco2 permeability	-0.081	0.326	0.9	log Papp in 10^{-6} cm/s
	Intestinal absorption	43.374	79.898	93.387	% Absorbed
Distribution	VDss	-1.855	0.709	0.094	log L/kg
	BBB permeability	-1.102	-0.954	-0.71	log BB
	CNS permeability	-3.74	-2.316	-2.048	log PS
Metabolism	CYP2D6 substrate	No	No	No	Categorical (Yes/No)
	CYP3A4 substrate	No	No	No	Categorical (Yes/No)
	CYP2D6 inhibitior	No	No	No	Categorical (Yes/No)
	CYP3A4 inhibitior	No	No	No	Categorical (Yes/No)
Excretion	Total Clearance	0.518	0.569	0.151	log ml/min/kg
	Renal OCT2 substrate	No	No	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose	0.7	0.42	0.478	log mg/kg/day
	Oral Rat Acute Toxicity	2.218	2.338	2.268	mol/kg
	Hepatotoxicity	No	No	No	Categorical (Yes/No)

Figure 5: ADMET characters of *M. calabura* leaves bioactive compounds, especially gallic acid, kaempferide, and genistein.

M. calabura leaves extracts was shown to lower the proportion of $CD4^+IFN\text{-}\gamma^+$ cells at all dosages. The reduction in expressed cytokine levels caused by *M. calabura* leaves extracts treatment did not differ substantially between dosages. All three substantially reduced the relative number of $CD4^+IFN\text{-}\gamma^+$ cells closer to normal. Quercetin in the extract could block the activation of IKK and NF-κB, reducing the ability of NF-κB to initiate IFN-γ synthesis [67]. This substance effectively suppresses the activation of the STAT1 signaling pathway, reduces the production of interferon-gamma (IFN-γ), and prevents T helper cells from differentiating into the Th1 subtype. Also, it directly affected IL-12-induced tyrosine phosphorylation and activation of JAK2 and TYK2 kinases, thereby inhibiting the activation of STAT3 and STAT4 transcription factors in Th1 cells, reducing their ability to synthesize

IFN-γ [68,69]. This mechanism is also shared with kaempferol, daidzein, and genistein in their activity to inhibit the IFN-γ synthesis in Th1 cells [69–71].

The effect of M. calabura leaves *extracts on TNF-α$^+$ expressing CD4$^+$ Th1 cells*

In this present study, we demonstrated that the high dose of leave extracts could slightly promote the TNF-α expression into the normal condition (Figure 6B and 6D). TNF-α is a pro-inflammatory cytokine produced mostly by T cells and macrophages that promotes inflammation by primarily serving as an inflammatory mediator in response to infection or other stressors by TNFR-I via NF-κB and AP-1 [46]. It is a pro-inflammatory cytokine that has been linked to various inflammatory disorders. The overexpression of TNF-α could lead to persistent inflammation and autoimmune diseases [72].

High glucose serum in hyperglycemia could induce a higher level of glycolysis and overproduction of ROS by the mitochondria, which then directly activate PKC, promoting activation of IKKα and IKKβ, subsequently activating NF-κB function as a transcription factor for TNF-α expression [73, 74]. This cascade will trigger the upregulation of TNF-α in Th1 cells and other myeloid lineage cells. A high concentration of TNF-α generated by these cells also acts as a positive feedback loop, triggering an even higher synthesis of TNF-α through a specific mechanism. The secreted TNF-α activates RIP1/MAP3K7 pathway upon recognition by the TNF-R1. The activation then promotes the phosporilation of IKKα and IKKβ, leading to the same downstream cascade to induce TNF-α expression in Th1 cells [72, 74-77]. Increased TNF-α expression can boost lipid synthesis through the lipogenesis via JNK and PI3K/Akt pathway and directly correlate to increased body weight [78–80]. Overexpression of TNF-α has also been related to the number of insulin-producing pancreatic cells by activating the apoptotic pathway to pancreatic β-cells via the mitochondrial apoptosis pathway involving STAT-1 [81], further limiting the production of insulin to lower the blood glucose level, making the condition persists and even worsen. Activating PI3K/Akt pathway by TNF-α synthesized by Th1 cells could also induce insulin resistance in essential tissues [82].

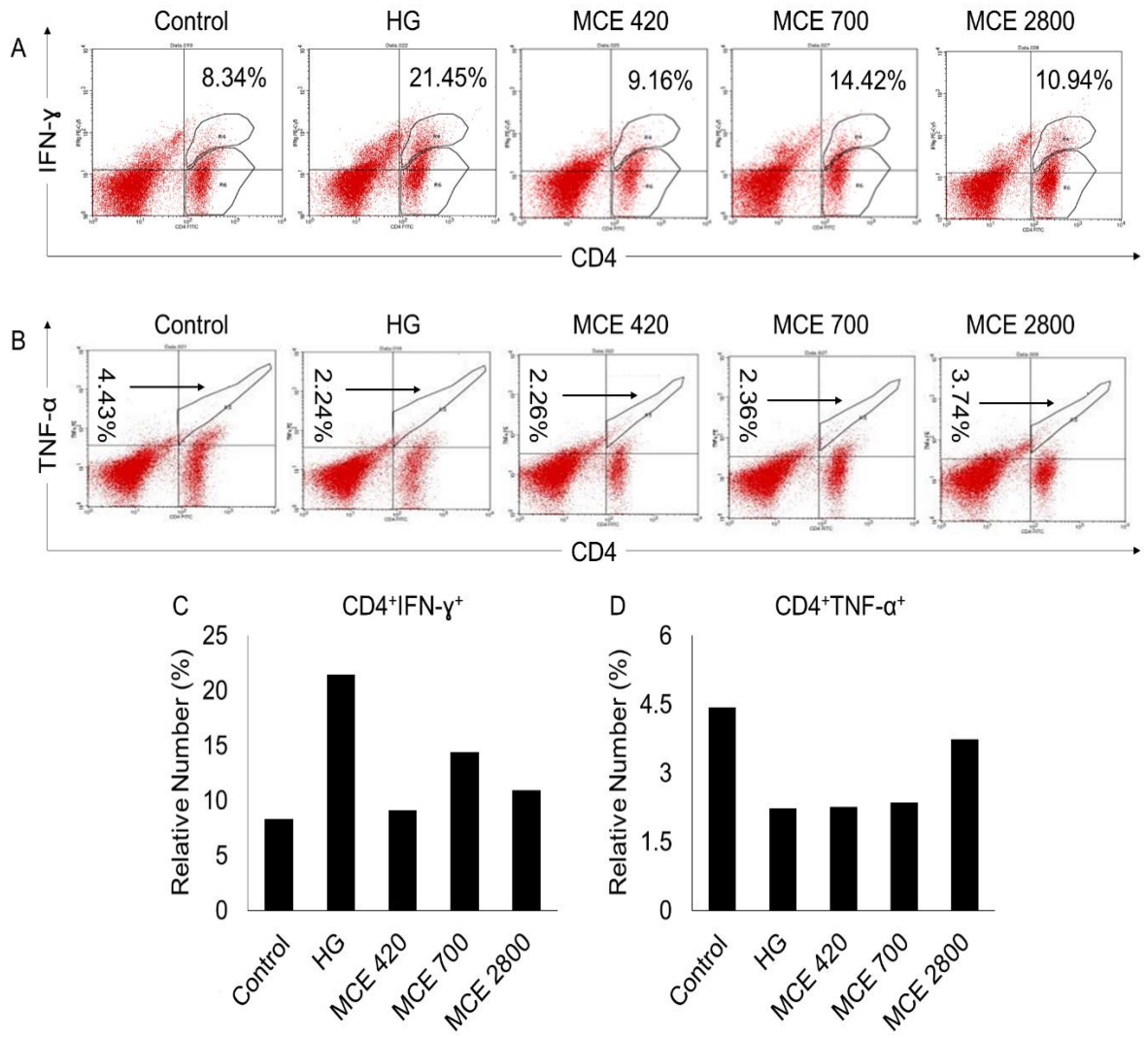

Figure 6: Modulation effects of *M. calabura* leaves extracts toward IFN-ɣ expressing $CD4^+$ Th1 cells (A and C) and TNF-α expressing $CD4^+$ Th1 cells (B and D) in high fat diet-administrated mouse into normal condition.

Administering *M. calabura* leaves extracts to the hyperglycemic mice model at all dosages resulted in a substantial reduction in TNF-α expression compared to the hyperglycemic control. The level of TNF-α in *M. calabura* leaves extracts treatment groups was comparable to healthy control mice. The reduction in TNF-α expression following administration of *M. calabura* leaves extracts was assumed to be due to flavonoids in the extract. Quercetin, for instance, is thought to inhibit TNF-α expression at the transcription level by inhibiting phosphorylation of Iκβα and Iκββ, hampering the activation of NF-κβ needed to its expression in Th1 cells [67, 83]. It also blocks the nuclear translocation of p50 and p65 subunits NF-κβ needed for transcription and the phosphorylation of JNK and ERK pathways, which are upstream of TNF-α expression pathway [67]. Kaempferol, genistein, and gallic acid are also thought to inhibit the overexpression of TNF-α in Th1 cells with a similar mechanism to quercetin, which is similar to the inhibition of IL-1β synthesis in the previous chapter [56–59]. They inhibit the initiation of TNF-α synthesis and response to TNF-α stimulation because it is a part of a positive feedback loop with a shared pathway [84]. The

administration of *M. calabura* leaves extracts containing those bioactive compounds could alleviate insulin resistance and pancreatic β-cell damage caused by hyperglycemia.

Conclusion

Almost all tested doses demonstrated the suppressive effect on pro-inflammatory cytokines production by macrophages and T-cell. This study demonstrated that *M. calabura* leaves extracts might play important role in reduce the immune system activity in high-fat diet-administrated mice model. However, there are many limitations which might need further investigation, especially on some subset of immune cells which play role in pro or anti-inflammatory response. Additionally, the effect of *M. calabura* leaves extracts on several biomarker related to hyperglycemia need to evaluate.

Conflict of Interest

No conflict of interest

Acknowledgement

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