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Research Article

Ameliorative Effect of Jamaican Cherry (*Muntingia calabura* L.) Leaf Extract Toward Glucose Control and Immune Cells Modulation in High Fat Diet-Administrated Mice

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Abstract: Hyperglycemia is a dangerous condition in which too much glucose circulates in the blood plasma and is the leading cause of diabetes mellitus. It is a complex condition with varying degrees that can change over time, mainly owing to metabolic factors that reduce insulin secretion, decrease glucose use, and increase glucose production. This study aims to evaluate *Muntingia calabura* leaf extract's effect on glucose control and immune cell modulation in high-fat diet-administrated mice. According to the result, we found that *M. calabura* leaf extract significantly reduced the fasting blood sugar. Importantly, *M. calabura* leaf extract exerts immunomodulation effects by suppressing the relative number of regulatory T cells in the hypoglycemic mice model. Finally, this study showed *M. calabura* leaf extract exerts ameliorative potency against hyperglycemia by lowering the blood sugar level and suppressing the regulatory T cells. These results suggested that *M. calabura* leaf extract could develop into complementary and alternative medicine.

1. Introduction

Hyperglycemia, or high blood sugar, is a dangerous condition in which too much glucose circulates in the blood plasma (Chaudhury et al., 2017; Wanrooy et al., 2018). It is a complicated disease with multiple degrees that can alter over time, primarily due to metabolic variables that diminish insulin secretion, decrease glucose utilization, and increase glucose generation (Ormazabal et al., 2018; Garcia et al., 2020). Specifically, hyperglycemia is defined as a condition in which the blood sugar level is consistently between 5.6 and 7.0 mmol L^{-1} (100-126 mg dl^{-1}), whereas diabetes is defined as a condition in which the blood sugar level is greater than 7.0 mmol L^{-1} (126 mg dl^{-1}) (American Diabetes Association Professional Practice Committee 2021). Endocrine abnormalities, end-stage terminal disease, significant

operations, and a prolonged unhealthy lifestyle such as excessive high-calorie eating, being overweight, and high amounts of stress are all common risk factors for acquiring this syndrome (Abideen et al., 2017; Escott et al., 2021; Riddle et al., 2021; Zhang et al., 2021).

Diabetes and hyperglycemia are frequently referred to as the same thing and used interchangeably. In fact, hyperglycemia is the most commonly diagnosed cause of diabetes, both type I and type II. Type I diabetes is characterized by decreased insulin production by pancreatic β-cells due to an autoimmune response driven by B cells and particular antibodies such as anti-islet cells, anti-GAD, and anti-insulin antibodies. It dramatically reduces insulin levels recognized by the insulin receptor in cells and downregulates the expression of glucose transporter protein in membrane cells, lowering glucose absorption by cells and increasing glucose concentration in the plasma, commonly known as hyperglycemia (Barnett, 2018; Basu et al., 2020). Type II diabetes, on the other hand, is characterized by insulin resistance associated with metabolic syndrome, resulting in irresponsive insulin receptors and their respective pathways, decreased expression of glucose transporters, and significantly reduced glucose intake by cells, leaving a high amount of glucose in the blood plasma. This hyperglycemic condition stimulates the pancreatic β-cells to produce more elevated insulin, resulting in hyperinsulinemia and, over time, causing high stress to the pancreatic β-cells by releasing amyloid in the extracellular matrix, a condition known as amyloid deposition, which can damage the β-cells and lower insulin production (Kanatsuka et al., 2018; Galicia-Garcia et al., 2020).

Treatment of diabetes is essential for hyperglycemia treatment. Acute hyperglycemia caused by type I diabetes may usually be managed with early insulin injection; however, persistent hyperglycemia can be controlled with oral hypoglycemic medicine and healthy lifestyle changes (Silver et al., 2018). However, the medications utilized are commonly pharmaceuticals such as metformin, thiazolidinediones, sulfonylureas, and meglitinides, which can cause lactic acidosis, edema, hypoglycemia, and diarrhea in patients (Wang et al., 2017; Wu et al., 2017; Harsch et al., 2018; Salvatore et al., 2019). As a result, people are beginning to recognize the potential of medicinal herbs in curing ailments with few side effects. For people who are aware that long-term use of pharmaceutical medications may have serious adverse effects, medicinal plants are a great therapeutic choice (Putra and Rifa'i, 2019; Putra et al., 2021; Rahayu et al., 2022; Nurcholis et al., 2023). In developing nations, traditional medicine is the primary therapy for around 80% of the population. Plant extract accounts for around 85 percent of traditional medicine (Ahn, 2017; Jamshidi-Kia et al., 2018). Herbal medicine development is progressing in tandem with public knowledge of contemporary pharmaceuticals' health dangers and toxicity. Until recently, several plants were investigated for their potential medicinal advantages for various ailments. According to some studies, bioactive compounds such as thymoquinone from *Nigella sativa*, curcumin from *Curcuma longa*, and (S)-[8]-gingerol from *Zingiber officinale* can increase insulin levels, increase pancreatic islet immunoreactivity, reduce oxidative stress by acting as free radical scavengers, and upregulate glucose transporter 4 (GLUT4) protein, increasing glucose transport activity (Noipha and Ninla-Aesong, 2018; Abdelkader et al., 2020; Den Hartogh et al., 2020).

One of the species gaining popularity as a therapeutic herb is *M. calabura* (Elaecarpaceae), sometimes known as cherry. In many places, cherry plants are employed as herbal treatments. In Peru, for example, flowers and tree trunks are used as antiseptics, while the leaves are cooked and used to treat prostate gland enlargement (Sarojini and Mounika, 2018). Cherry leaf treats stomach problems, acne, and chickenpox in the Philippines (Tantengco et al., 2018). Cherry leaves have been examined for quite some time in Indonesia as a traditional remedy for treating diabetes. Based on chromatography examination, it contains various bioactive components that may contribute to its anti-hyperglycemic activity, including geniposide, luteolin, daidzein, quercetin, kaempferol, formononetin, 6-hydroxy flavone, gallic acid, kaempferide, genistein, and chrysin (Zakaria et al., 2019; Zolkeflee et al., 2022). However, little study has been undertaken to determine the specific effect of cherry leaf on hyperglycemia or diabetes. Therefore, more research into the advantages of cherry leaf on these entities is required. Thus, this study aims to evaluate the effect of *M. calabura* leaf extract on glucose control and immune cell modulation in high fat diet-administrated mice.

2. Material and Methods

2.1. *M. calabura* leaf extraction procedure

Dry powder of cherry leaf was obtained from Materia Medica, Batu, East Java. Decoction with freeze-drying methods was used for cherry leaf extraction. The cherry leaf powder was then cooked in distilled water (80°C) for about two hours with a leaf: aquadest ratio of 1: 10 (gr: ml) until the final volume was half the original volume, then filtered with a thin and clean cloth. The filtrate was then placed in a freezer set to -70 °C before freeze-dried to eliminate moisture.

2.2. Experimental treatments

Approximately 20 three-week-old male BALB/c mice were used. These pathogen-free mice were obtained from Gadjah Mada University, Yogyakarta. The mice were split into five groups with four repetitions, including vehicle group (aquadest), hyperglycemic group (12 weeks of HFD), MCE420 (12 weeks of HFD + 2 weeks of cherry leaf extract with 420 mg kg^{-1} BW), MCE700 (12 weeks of HFD + 2 weeks of cherry leaf extract with 700 mg kg^{-1} BW), and MCE2800 (12 weeks of HFD + 2 weeks of cherry leaf extract with 2800 mg kg^{-1} BW). The variation of doses used in this study was based on our preliminary study. HFD feed consists of 35% Hi-Gro 551, 10% high protein flour, 30% liquid fructose, 8% duck egg yolk, and 17% oil generated from beef fat (Saravanan and Pari, 2015). All ingredients are mixed and formed into a biscuit, baked at 100 °C for 10 minutes. The glucose levels of all mice were assessed on the tenth week, and then they were administered with *M. calabura* leaf extract (MCE) when the blood sugar levels had reached higher than 140 mg dL^{-1} after underwent fasting for 12 hours for two weeks. This research has been evaluated and received approval by the Research Ethics Committee of Brawijaya University, Malang with no. 670-KEP-UB for conducting animal model experiment.

2.3. Blood sugar test and evaluation

Blood sugar levels were measured using the GlucoDr AGM-2100 after the mice were fasting for 12 hours, and blood was drawn from the tail end and dripped on the stick of the blood sugar check instrument. Hyperglycemia occurs when fasting blood sugar levels exceed 140 mg dL^{-1} (Maffettone et al., 2018; Goyal et al., 2020). Blood sugar levels were measured twice after the week-10 of HFD administration and once after week-12 of therapy.

2.4. Splenocytes isolation

After two weeks of oral administration of *M. calabura* leaf extract, the mice were sacrificed to isolate the splenocytes. The spleen was then washed with PBS and homogenized in PBS using a syringe in a clockwise motion. After that, the homogenate was placed in a propylene tube and replenished with PBS until the amount reached 10 ml. The sample was then placed in a propylene tube and kept in an icebox until further analysis. The spleen homogenate was then centrifuged at 2500 rpm for 5 minutes at 10 °C. The pellets were collected and resuspended in 1 ml of PBS.

2.5. Flow cytometry analysis

In this study, we employed the FACS procedures and analyses according to our previous study (Putra et al., 2015; Putra et al., 2016). About 50 μl of pellet suspension of splenocytes was placed in a microtube containing 500 μl of PBS and centrifuged at 2500 rpm for 5 minutes at 4 °C. Extracellular staining was performed by adding 50 μl of extracellular antibody to the resultant pellet and incubating it at 4 °C for 20 minutes. It was then resuspended in 300 μl of PBS and put in a cuvette for flow cytometry analysis. After extracellular labeling, intracellular staining was performed using a suspension of cytofix-cytoferm and incubated for 20 minutes at 4 °C. Washperm was then applied in up to 500 μl and centrifuged for 5 minutes at 2500 rpm at 10 °C. The pellets were then resuspended in 300 μl of PBS after being treated with 50 μl of intracellular dye. The suspension is then placed within the cuvette. The FACS Calibur^{TM} Flow cytometer (BD Bioscience) was used for the analysis. The extracellular dyes used were FITC-labeled rat anti-mouse CD4, PE-labeled rat anti-mouse CD25, and PE/Cy5-labeled rat anti-mouse TGF-β.

2.6. Compounds and Protein-Protein Interactions Prediction

About three compounds that are widely found in *M. Calabura,* including kaempferide, genistein, and gallic acid were evaluated in this study. In our previous study, the STITCH webserver (http://stitch.embl.de/) was used to predict the chemical association network toward the proteins and its specific biological activity (Putra et al., 2017; Putra et al., 2023).

2.7. Statistical analysis

The data were analyzed with the normality and homogeneity tests of variance. The CellQuest^{TM} (BD Bioscience) and SPSS 20.0 for Windows were used to examine the data, then assessed using an ANOVA test with a 95% confidence interval, followed by a Tukey's honest significant difference test.

3. Results and Discussion

3.1. *M. calabura* leaf extract reduces blood sugar

In this study, we evaluate the effect of MCE on the blood sugar level and immune modulation in HFD-administrated mice (Figure 1). Following a high sugar and fat meal, glucose levels in mice's blood, and body weight increased dramatically compared to normal mice (Figure 2). According to those findings, these mice groups displayed signs of obesity, indicating excessive lipid buildup in adipose tissue. Overnutrition causes adipocyte hypertrophy and hyperplasia, which leads to cellular stress, which triggers inflammatory responses in adipose tissue (Longo et al., 2019).
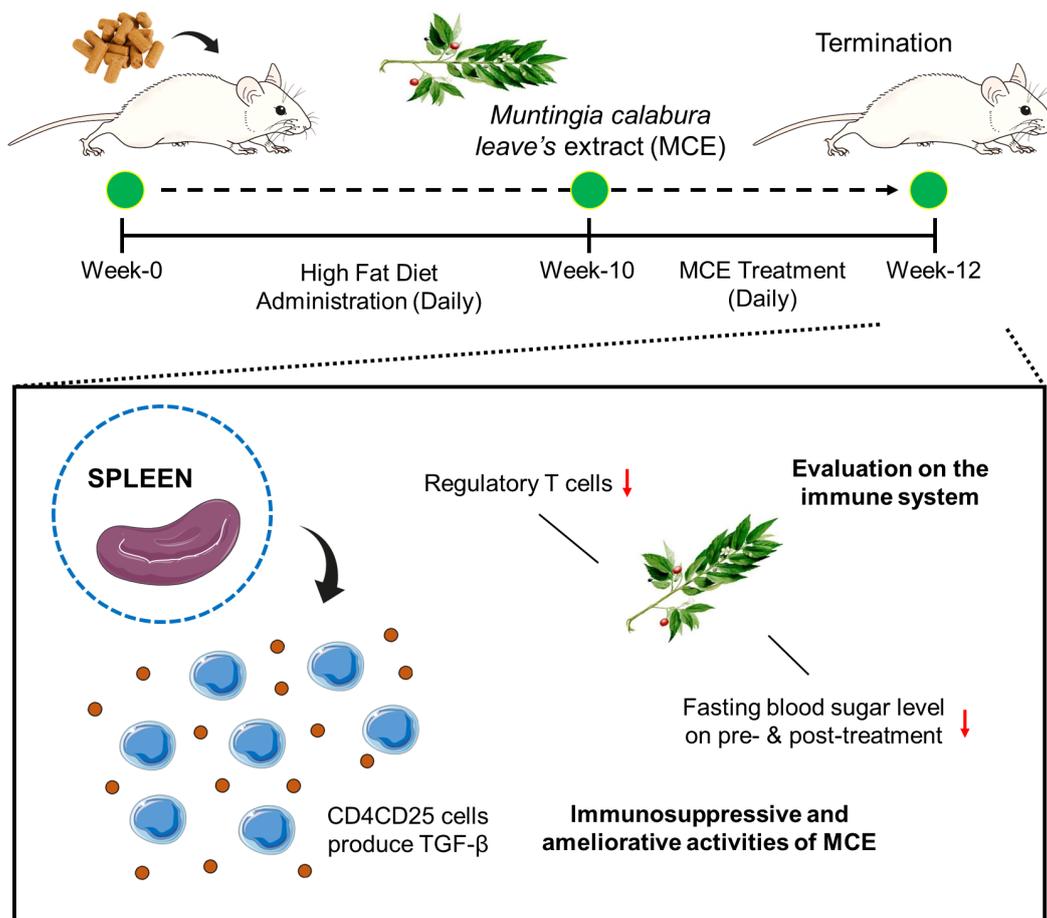

Figure 1. Schematic picture showed how *M. calabura* leaf extract suppresses the regulatory T cells and fasting blood sugar of high-fat diet-administrated mice to normal conditions.

Inflammatory reactions in adipose tissues self-generate, resulting in elevated local and systemic levels of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1 (Ellulu et al., 2017). These pro-inflammatory cytokines connect to cell-surface receptors and activate kinases such as JNK, which phosphorylate IRS-1 on serine residues, limiting its action. Thus, inflammatory cytokines suppress the insulin signal, resulting in insulin resistance, since irresponsive insulin receptors and their corresponding pathways reduce the expression of glucose transporters and significantly reduce glucose absorption by cells, resulting in hyperglycemia. TLR2 and TLR4 activate the kinase proteins JNK and IKK-β, boosting the production and release of pro-inflammatory cytokines and blocking the insulin signaling pathway, resulting in hyperglycemia (Chen et al., 2015; Yung and Giacca, 2020).

Hyperglycemia promotes glycolysis and tricarboxylic acid cycle fluxes, which raise $NADH/NAD^+$ ratios in the cell's cytosol and mitochondria. This increases electron disposal at the electron transport chain, producing reactive oxygen species (ROS) (Ola, 2021). Increased ROS levels in the cells would activate the JNK pathway while simultaneously inhibiting the insulin signaling cascade, resulting in high glucose accumulation in the blood because the cells do not respond appropriately to insulin communication signals and cannot easily take glucose from the blood (Volpe et al., 2018). It has also been proposed that high concentrations of IL-1β, IFN-γ, and TNF-α might interfere with insulin sensitivity (Tao et al., 2019; Wondmkun, 2020) and that increasing high oxidative stress in pancreatic β-cells and cell death by activation of the caspase-9 pathway could further impair insulin levels (Eguchi et al., 2021). High-fat content in HFD may also activate TLR4-mediated pro-inflammatory signaling pathways in pancreatic cells by activating NF-κB and translocating into nuclei, upregulating TNF-β expression, and initiating the activation of receptor-interacting protein 3 (RIP3) kinase, which marks the activation of programmed necrosis (Meng et al., 2015; Hong et al., 2020).
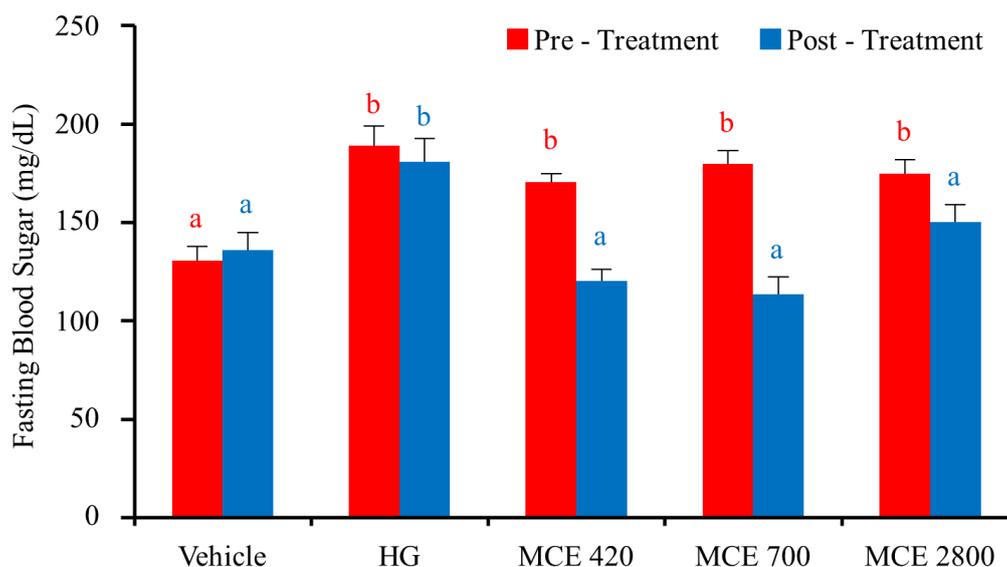

Figure 2. Fasting blood sugar level on pre- and post-treatment of *M. calabura* extract in high fat diet-administrated mice. The p value < 0.05 was considered significant. The graph shows considerable differences using alphabetic characters. Hyperglycemic group (HG); *M. calabura* leaf extract (MCE).

Additionally, to validate our findings, we predicted the main compounds of *M. calabura* leaf such as genistein, kaempferide, and gallic acid. According to the prediction, these compounds are included in many biological processes, including cellular response to lipids, glucose homeostasis, positive regulation of fat cell differentiation, and positive regulation of nitric oxide biosynthetic process (Figure 3). Several investigations indicate that *M. calabura* leaf extract has a high quantity of geniposide, pinostrobin, genistein, daidzein, quercetin, kaempferol, formononetin, and gallic acid (Zakaria et al., 2016; Zakaria et al., 2019; Zolkeflee et al., 2022). Geniposide reduces hyperglycemia-induced oxidative stress and inflammation by upregulating the Nuclear factor erythroid 2-related factor 2 (NRF2) pathway, inhibiting ROS buildup and decreasing NF-κB activation and the consequent inflammatory response

(Tu et al., 2021). It is also thought to suppress the transcriptional activity of Forkhead box protein O1 (FOXO1) by activating the phosphorylation of protein kinase B (PKB) (Yang et al., 2018), preventing more glucose from passing through the blood plasma. Daidzein helps to increase the mRNA level in β-cells to exert more insulin production (Zolkeflee et al., 2022), increasing the ratio of GLUT4 to Na^+/K^+ ATPase in the plasma membrane, which increases glucose uptake (Das et al., 2018), upregulating the gene expression of Peroxisome proliferator-activated receptor gamma (PPARγ) and adiponectin and downregulating the monocyte chemoattractant protein-1 (MCP-1) and TNF-α gene expression in adipose (Sakamoto et al., 2014). Quercetin protects the intact-cells of the islets of Langerhans by preventing lipid peroxidation and scavenging free radicals, hence maintaining appropriate insulin concentration (Abdelkader et al., 2020). Genistein reduced the levels and mRNA expression of pro-inflammatory cytokines IL-1, IL-6, and TNF-α, and when combined with gallic acid, it suppressed the ROS/Akt/NF-κB pathway and increased adenosine monophosphate protein kinase (AMPK) activation, enhancing insulin sensitivity (Xu et al., 2021; Goh et al., 2022).

3.2. *M. calabura* leaf extract suppresses the relative number of CD4$^+$CD25$^+$ T Cells

Regulatory T cells (Tregs) are a subpopulation of CD4$^+$ T cells that secrete TGF-β, IL-10, and IL-4 to prevent autoimmune and pro-inflammatory responses to preserve peripheral tolerance and decrease antigen-specific immune responses carried out by CD8$^+$ T cells (Qiao et al., 2016; Zhou et al., 2021). In hyperglycemic mice, the relative number of CD4$^+$CD25$^+$ Tregs rose considerably compared to normal mice (Figure 4). It is suggested that diabetic mice's spleen CD4$^+$CD25$^+$ Tregs have defective immunosuppressive capability despite having a higher relative number, which is thought to correlate with decreasing insulin levels because low insulin levels in hyperglycemic mice impacted thymic CD4$^+$CD25$^+$ Treg development. Furthermore, Treg cells are dominated by a subpopulation that lacks the CD62L protein (Putra and Rifa'i, 2020). They exhibit high levels of CD44 and CTLA-4 expression simultaneously, which weakens their suppressive action (Zhen et al., 2012; Hyun et al., 2019), hinders continuous cell activation, and boosts pro-inflammatory cytokine production, which is predominantly carried out by T regulatory type 1 cells (Hull et al., 2017).

Under hyperglycemic conditions, increased production of pro-inflammatory cytokines such as IL-2 by adipocytes, monocytes, and macrophages might occur due to high blood glucose levels and decreased insulin expression, which leads to increased CD25 expression in the CD4$^+$ T cell population (Kochumon et al., 2020). As previously demonstrated, high glucose levels can enhance the production of pro-inflammatory cytokines via many pathways, including the NF-κB pathway (Meng et al., 2015; Hong et al., 2020). Fatty acids can alter T cell functions such as proliferation and cytokine production, hence increasing inflammation. Low-dose fatty acids, in contrast to large dosages, can enhance T cell growth and alter the production of cytokines such as TNF-α, IL-6, IL-8, IL-1, IL-2, IL-10, and IFN-γ (Heintzman et al., 2022). We anticipated that a highly inflammatory milieu, namely a high concentration of IL-2, might convert naive CD4$^+$ cells to CD4$^+$CD25$^+$ Treg cells, which then counteract those highly inflammatory circumstances by releasing large quantities of anti-inflammatory cytokines, including TGF-β, creating a positive loop that promotes additional Treg cells, increasing their relative proportion.

The administration of cherry water extract to hyperglycemic mice at all experimental doses lowered the relative number of CD4$^+$CD25$^+$ regulatory T cells to near-normal levels. All doses had no significant change (Figure 4), with all three significantly reducing the relative number of regulatory T cells. The decrease in the close number of CD4$^+$CD25$^+$ T cells may be related to a reduction in CD25 expression in the CD4$^+$ T cell population induced by a high content of quercetin (Kobori et al., 2016), which is consistent with the results of (Leyva-López et al., 2016), who discovered that flavonoids like quercetin could reduce the activity of NF-κB, a common transcription factor for pro-inflammatory cytokines that are activated by ROS, resulting in suppression of pro-inflammatory Furthermore, the extract's kaempferol may boost and maintain forkhead box P3 (FOXP3) expression in Treg cells while decreasing proto-oncogene serine/threonine-protein kinase (PIM-1)-mediated FOXP3 phosphorylation, improving their suppressive action on effector T cells and pro-inflammatory helper T cells (Lin et al., 2015).
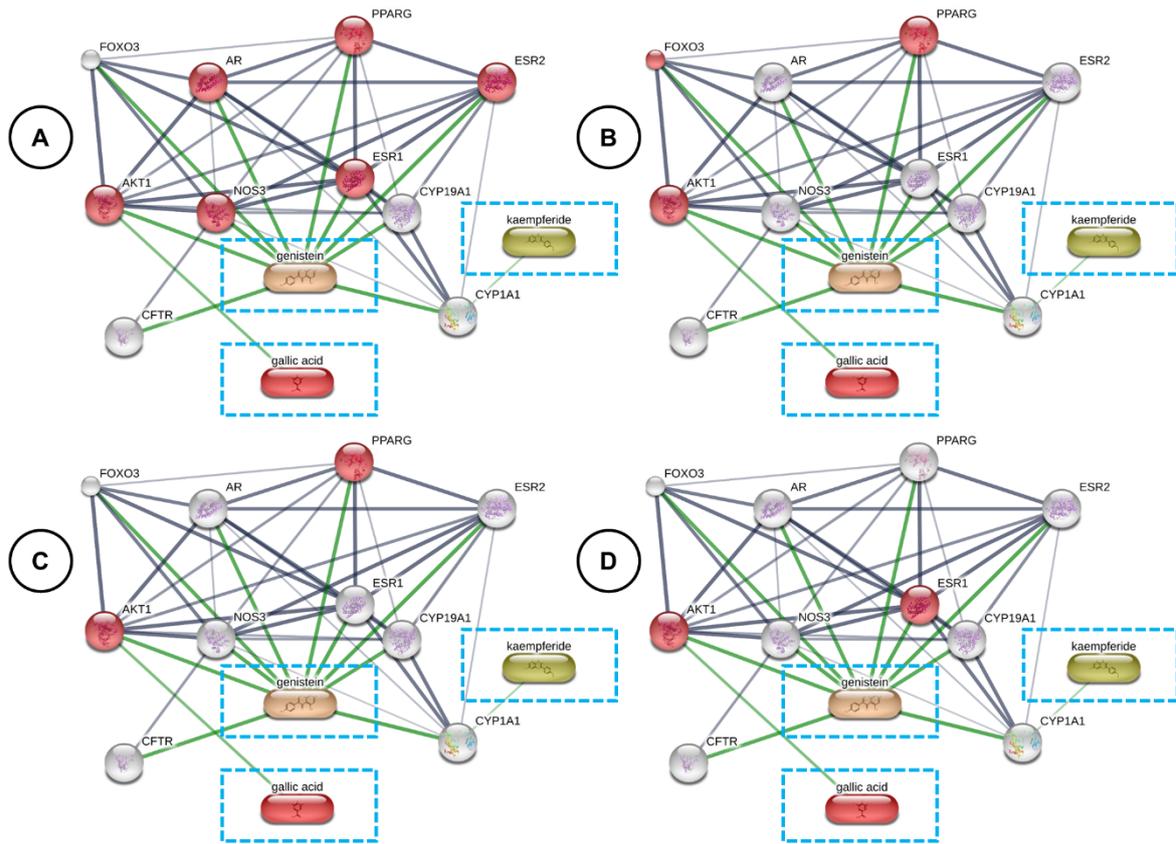
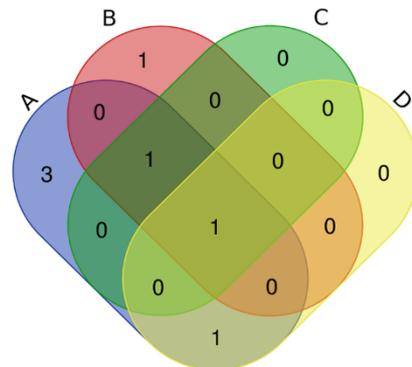| Symbol	Biological Process	False Discovery Rate	Protein Involved
A	Cellular response to lipid	6.74E-05	AKT1, AR, ESR1, ESR2, NOS3, PPARG
B	Glucose homeostasis	0.0199	AKT1, FOXO3, PPARG
C	Positive regulation of fat cell differentiation	0.0449	AKT1, PPARG
D	Positive regulation of nitric oxide biosynthetic process	0.0283	AKT1, ESR1

Figure 3. The *M. calabura* compounds-proteins interaction network analysis. (A). Cellular response to lipid; (B). Glucose homeostasis; (C). Positive regulation of fat cell differentiation; (D). Positive regulation of nitric oxide biosynthetic process.

It has been proposed that enhanced circumstances in the cherry treatment groups may function as a negative feedback mechanism. Fewer Treg cells are required to limit and stabilize lower pro-inflammatory cytokine expression and population pro-inflammatory immune cells in adipose tissue and the pancreas. Furthermore, in the untreated hyperglycemic group, a lower number of Tregs with highly suppressive characteristics is preferable to a higher number of Tregs with low suppressive action because these highly suppressive Tregs are more capable of alleviating tissue-damaging inflammatory responses than their less-suppressive counterparts. According to the previous explanation, numerous cherry extract components enhance the targeted tissues' inflammatory milieu and considerably ameliorate the hyperglycemic condition. Thus, lowering the oxidative stress reduces the Tregs needed to control the inflammation caused by hyperglycemia.

3.3. *M. calabura* leaf extract suppresses the relative number of TGF-β-expressing CD4⁺CD25⁺ T Cells

The number of CD4⁺CD25⁺ T lymphocytes expressing TGF-β rose considerably in hyperglycemic mice compared to normal mice because hyperglycemia increases the production of TGF-β and its receptors to counteract the buildup of ROS as well as chronic inflammation (Budi et al., 2015; Chen et al., 2020). Increased blood sugar levels mediate these effects via various pathways, including the polyol activation pathway, protein kinase C, and severe oxidative stress (Jha et al., 2016; Dhanya, 2022). As previously noted, hyperglycemia may increase ROS levels in mouse tissue due to greater blood glucose levels and reduced insulin synthesis by pancreatic β-cells.
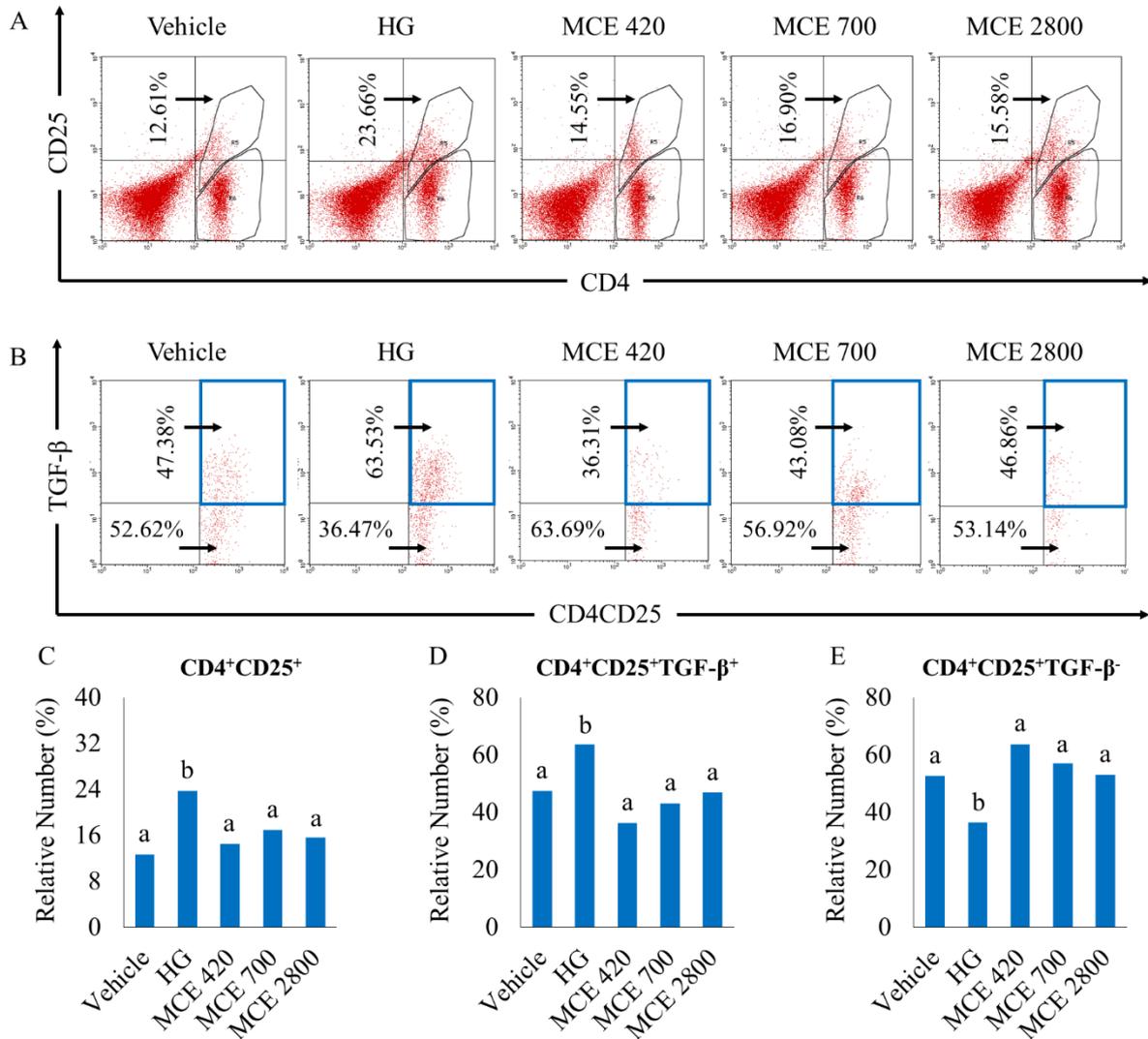

Figure 4. Immunosuppressive effects of *M. calabura* extract toward regulatory T cells high-fat diet-administrated mice. The p-value < 0.05 was considered significant. The graph shows considerable differences using alphabetic characters. Hyperglycemic group (HG); *M. calabura* leaf extract (MCE).

Furthermore, increasing fatty acid levels might increase ROS generation and decrease catalase activity in T cells, resulting in cell death (De Jong et al., 2014). These assertions are consistent with the findings, which reveal a direct relationship between mouse body weight and a rise in CD4⁺CD25⁺TGF-β⁺ T cells. Higher oxidative stress imposed by hyperglycemia-induced inflammation in pancreatic β-cells might cause cell death, as discussed in the preceding chapter, via endoplasmic reticulum stress and mitochondrial malfunction. Another critical issue is that pancreatic β-cells have decreased antioxidant activity, rendering them more vulnerable to oxidative stress and necrosis (Eguchi et al., 2021). If not mediated by the insulin promoter, TGF-β might increase β-cell death and pancreatic fibrosis via the

TGF-β/Smad3 signaling pathway (Lee et al., 2021). Higher TGF-β expression in the untreated hyperglycemia group might alternatively be interpreted as an attempt to increase insulin production from damaged β-cells, putting additional strain on this already harmed tissue (Dhawan et al., 2016).

We discovered that all three dosages significantly lowered the quantity of TGF-β generated by regulatory T cells (Figure 4). The three doses provided did not differ much since they were labeled the same way, yet all three dramatically lowered cytokine levels compared to untreated hyperglycemic mice. Aside from the previously mentioned impact, we believe bioactive chemicals in cherry leaf extract may block TGF-β production in Treg cells. To the best of our knowledge, bioactive substances like quercetin, kaempferol, and genistein might block PKC activation and its signal transduction pathway, hence suppressing TGF-β expression in Treg cells (Kanazawa et al., 2017; Alam et al., 2020; Salehi et al., 2020; El-Far et al., 2022). TGF-β expression, on the other hand, appears to be strongly related to the relative amount of Treg cells and insulin production. As previously indicated, TGF-β upregulation is preceded by hyperactivation of its synthesis pathway, which is driven by hyperglycemia. Higher insulin levels in the treated groups may also help ameliorate hyperglycemia by reducing glucose levels and suppressing TGF-β synthesis and its response pathways.

Conclusion

This study showed *M. calabura* leaf extract exerts ameliorative potency against hyperglycemia by lowering the blood sugar level and suppressing the regulatory T cells. These results suggested that *M. calabura* leaf extract could develop into complementary and alternative medicine. However, more research on the effects of *M. calabura* leaf extract on various immune cells is required.

Acknowledgements

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