Benefits of *Coriandrum sativum* L. Seed Extract in Maintaining Immunocompetent Cell Homeostasis

(Kebaikan Ekstrak Biji *Coriandrum sativum* L. dalam Mengekalkan Homeostasis Sel Imunokompeten)

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disease followed by an increase in blood glucose and impaired metabolism of proteins, lipids and carbohydrates. In general, these conditions are caused by impaired insulin secretion and/or defects of insulin receptors. As yet, there is no effective drug capable of treating DM. Manifestations of DM worsen when accompanied by severe inflammation. Therefore, the avoiding strategy and management of inflammation in DM are the primary courses of action in preventing further damage. In this study, we had evidence that *Coriandrum sativum* L. extract (CSE) could be used as an alternative to relieve symptoms in DM mice model. Previously, CSE has been shown to be able to inhibit the synthesis of the pro-inflammatory molecule interleukin (IL)-6 by both B220 and CD11b cells. Here, we showed that CSE prevents over-activation of CD4 and CD8 T lymphocytes. The predominance of T cells expressing CD62L on both CD4 and CD8 T cells after administration of CSE indicated that there were obstacles to activation. CSE also suppressed excessive CD25 expression, causing CD25 expression in CD4 T cells to return to normal levels. Thus, this study showed the therapeutic activity of CSE in DM mice model by suppressing the pro-inflammatory cytokines, modulate the activation of naïve T cells, and maintain the population of CD4$^+$CD25$^+$ cells.

Keywords: *Coriandrum sativum* L.; diabetes mellitus; interleukin-6; pro-inflammatory

ABSTRAK

Diabetes mellitus (DM) adalah penyakit metabolik yang diikuti oleh peningkatan glukosa darah dan gangguan metabolisme protein, lipid dan karbohidrat. Secara umumnya, keadaan ini disebabkan oleh rembesan insulin yang terjejas dan/atau kecacatan reseptor insulin. Setakat ini, tiada dadah berkesan yang mampu merawat DM. Manifestasi DM bertambah teruk apabila disertai dengan keradangan yang teruk. Oleh itu, strategi mengelakkan dan pengurusan keradangan dalam DM adalah tindakan utama dalam mencegah kerosakan selanjutnya. Dalam kajian ini, kami mempunyai bukti bahawa ekstrak *Coriandrum sativum* L. (CSE) boleh digunakan sebagai alternatif untuk melegakan gejala dalam model tikus DM. Sebelum ini, CSE telah ditunjukkan dapat merencat sintesis molekul pro-radang interleukin (IL)-6 oleh kedua-dua sel B220 dan CD11b. Di sini, kami menunjukkan bahawa CSE menghalang pengaktifan berlebihan limfosit CD4 dan CD8 T. Penguasaan sel T yang mengekspreskan CD62L pada kedua-dua sel T CD4 dan CD8 selepas perlakuan CSE menunjukkan bahawa terdapat halangan untuk pengaktifan. CSE juga menindas ekspresi CD25 yang berlebihan menyebabkan ekspresi CD25 dalam sel T CD4 kembali ke tahap normal. Oleh itu, kajian ini menunjukkan aktiviti terapeutik CSE dalam model tikus DM dengan menekan sitokin pro-radang, memodulasi pengaktifan sel T naif dan mengekalkan populasi sel CD4$^+$CD25$^+$.

Kata kunci: *Coriandrum sativum* L.; diabetes mellitus; interleukin-6; pro-radang

INTRODUCTION

DM is a metabolic disease that causes high blood glucose. The disease is characterised by disorders of carbohydrate and fat metabolisms that are generally associated with inhibition of insulin secretion (Frederico et al. 2016; Hajiaghaalipour et al. 2015). According to the WHO (2018), the prevalence of DM has reached 592 million in 2035. DM is associated with a high risk of heart attack, blindness and kidney failure (Hackett & Jacques 2009; Sharma et al. 2016). Type-2 DM causes a decrease in the number of regulatory T cells, and this condition likely leads to an inflammatory state which contributes to the occurrence of diabetic complication (Harford et al. 2011; Qiao et al. 2016). Importantly, the pro-inflammatory metabolic conditions including chronic hyperglycemia and elevated cytokine circulatory levels suggest immune deterioration (Quan et al. 2015; Saxena et al. 2013). Further, the inability of β-pancreatic cells to produce insulin in type-2 DM is caused by a disruption in the regulation of glucose uptake via alterations to GLUT-4 protein function. Dysregulation of glucose uptake can increase the secretion of pro-inflammatory molecules such as TNF-α and interleukin-6 (IL-6) that cause tissue damage (Hajiaghaalipour et al. 2015).

Management of type-2 DM still relies on insulin to maintain quality of life and prevent ongoing damage (Kaku 2010). Prolonged use of anti-diabetes drugs causes liver and kidney damage (Nasri & Rafieian-Kopaei 2014). Currently, DM patients tend to choose herbal medicines that have fewer side effects. Food selection is one approach to prevent and manage DM and its complications (Bahadoran et al. 2013). Coriander seeds (*Coriandrum sativum* L.) are often claimed to be medicinal plants capable of treating digestive disorders, DM, rheumatism, and joint pain. Experiments using *C. sativum* L. as anti-diabetes drugs have been performed, but explanations concerning immune cells are still insufficient. Ethanolic extract of C. *sativum* seeds at a dose of 200 mg/kg body weight (BW) can increase pancreatic beta activity and decrease glucose, total cholesterol, and triglycerides (Aissaoui et al. 2011; Das et al. 2019a, 2019b; Naquvi et al. 2011). Coriander seed powder contains antioxidants such as flavonoids and polyphenols with the potential to reduce lipid peroxide levels. Flavonoids have a role in improving pancreatic β cell proliferation, stimulating insulin secretion, reducing inflammation and maintaining blood glucose balance in adipose tissue, while polyphenols have a role in increasing the activation of liver enzymes associated with glycogenesis and fat metabolism (Bahadoran et al.

2013; Deepa & Anuradha 2011; Hajiaghaalipour et al. 2015; Hossain et al. 2016; Prachayasittikul et al. 2018). In this study, we demonstrated that *C. sativum* inhibited the activation of immunocompetent cells to prevent the production of excessive pro-inflammatory molecules.

MATERIALS AND METHODS

MOUSE AND DM TYPE 2 INDUCTION

This study was conducted using 9-week-old male BALB/c mice maintained in a pathogen-free facility in the Biology Department of Brawijaya University. The experimental protocol had been granted Brawijaya University's Ethical Clearance from the Research Ethics Committee (reg. no. 670-KEP-UB). Food and drink were given *ad libitum* and the mice were maintained in optimal conditions in the animal center of Brawijaya University. Mice were maintained on a 12 h dark/light cycle. Diabetic induction was performed by intraperitoneal injection with streptozotocin (STZ; 145 mg/kg BW) in a method was adopted from Deepa and Anuradha (2011) and Furman (2015) with modifications. Blood glucose level five days post-induction was 200 mg/dL, whereas in normal controls it was 130 mg/dL. If fasting blood glucose level (FBG) was greater than 140 mg/dL, mice were considered to have hyperglycemia (Clee & Attie 2007).

C. sativum EXTRACT (CSE)

C. sativum seed extraction was performed according to Sithisarn et al. (2015) with modification. Briefly, 100 g of coriander powder was immersed in 1 L of 96% ethanol and shaken three times before decanting the supernatant. Each bout of shaking was performed for 24 h. The supernatant was then evaporated with an evaporator device to obtain a condensed extract. This extract was administered to diabetic mouse models at a dose of 25, 50 and 100 mg/kg BW.

LIQUID CHROMATOGRAPHY-HIGH-RESOLUTION MASS SPECTROMETRY (LC-HRMS)

Coriander extract in paste form was diluted according to standard methods using certain solution. An optimal viscosity was achieved for the sample with a final volume of 1300 μL. The sample was then vortexed for 1 min and centrifuged for 2 min at 600 rpm. The supernatant was decanted and filtered through a 22 μm filter and placed in a cuvette. The sample was then fed into the LC-HRMS machine.

ANTIBODIES

There were several antibodies used in this study to detect and evaluate the immune cells or cytokines involved in the diabetic mechanism. Moreover, we provided numerous types of antibodies including FITC-conjugated CD4 anti-mouse (clone: GK1.5), CD8a anti-mouse PE (clone: 53-6.7, BioLegend), CD62L anti-mouse PE/Cy5 mouse (clone: MEL-14, BioLegend), CD25 anti-mouse PE (clone: 3C7, BioLegend), IL-6 PE/Cy5.5-conjugated anti-mouse (NBPI clone, purchased from Novus Biologicals LLC), phycoerythrin (PE)-conjugated anti-mouse B220 (clone RA3-6B2, purchased from BD Pharmingen) and FITC anti-mouse/human CD11b (clone: M1/70, BioLegend).

CSE ADMINISTRATION AND CELL ISOLATION

In this study, CSE was administered orally at different doses (25, 50, and 100 mg/kg BW) in DM mouse models once daily for 2 weeks. After that, the mice were sacrificed, and the spleen cells were isolated by removing and washing spleen organ with sterile RPMI 1600 medium. The spleen was placed in a petri dish containing sterile RPMI 1600 medium and crushed using a syringe holder. A single cell suspension was centrifuged in 15 mL polypropylene tubes at 2500 rpm at 12 °C for 3 min. The supernatant was aspirated, and the pellet was resuspended in 1 mL of RPMI 1600 medium containing 10% FBS.

INTRACELLULAR CYTOKINE STAINING

In this study, intracellular cell staining was done in the presence of permeabilisation buffer. Cells (1×10^6) were incubated with antibodies suitable for cell surface molecule staining with anti-CD11b and anti B220. Intracellular molecule staining was done by incubating cells with the anti-IL-6 antibody. Antibodies were applied at a concentration of 5 µg/100 µL. In this experiment, we used spleen cells and the number of cells was adjusted as described above.

FLOW CYTOMETRIC ANALYSIS

Flow cytometric analysis was performed using FACS Calibur™ (BD-Biosciences, San Jose, CA) to quantify and evaluate the expression number of immune cells or cytokines production. Intracellular cytokine staining was performed according to the manufacturer's protocol using Cytoperm/Cytofix kit (BioLegend) according to our previous protocol (Rifa'i et al. 2014).

DATA ANALYSIS

We used BD Cell Quest PRO software to analyse data obtained from flow cytometry. The data were analysed by one-way ANOVA with a significance level of p-value < 0.05. Then, a post-hoc Tukey HSD (Honestly Significant Difference) test was performed to determine the difference when the ANOVA test showed significant results. Statistical analysis was performed by SPSS version 16 for Windows.

RESULTS AND DISCUSSION

CSE INHIBITED IL-6 EXPRESSION IN CD11B AND B220 CELLS

The outcome of LC-HRMS showed that the coriander extract contained abundant active compounds such as trigonelline, chlorogenic acid, 7-hydroxycoumarine, 4,5-dicaffeoylquinic acid and betaine (Figure 1). According to Yin et al. (2018), these two compounds can reduce islet cell apoptosis and improve pancreatic function in type-2 diabetic mice. In this experiment, we found that 2 weeks CSE administration in diabetic mouse models decreased blood glucose levels significantly from 495 to 316 mg/dL (p-value < 0.05). Consequently, CSE affected immunological status and physiological changes in treated mice (Frederico et al. 2016). In this experiment, we measured the production of the IL-6 in both CD11b and B220 cells. Normally, IL-6 is produced by all types of immune cells, including macrophages and stromal cells (Barros 2013; Hunter & Jones 2015). Flow cytometric analysis showed a decrease in IL-6 production in CD11b cells after receiving CSE (Figure 2). CD11b cells of DM mouse models produced high amounts of IL-6 compared to those treated with CSE. Around 16% of CD11b cells isolated from DM mouse models expressed IL-6, while in healthy mice, it was expressed by 6% of CD11b cells (CD11b+IL-6+). In general, CSE administration in DM mouse models could significantly decrease IL-6 production in CD11b cells (p-value < 0.05). CSE with a dose of 25, 50 and 100 mg/kg BW was able to reduce IL-6 production in CD11b cells. Interestingly, the inhibition of IL-6 synthesis occurred not only in CD11b cells but also in B220 cells. Around 14% of B220 cells of DM mouse models expressed IL-6 (B220+IL-6+), whereas B220 cells expressing IL-6 accounted for 10% of B220 cells in healthy mice. In the diabetic mouse model, IL-6 expression increases (Figure 3).
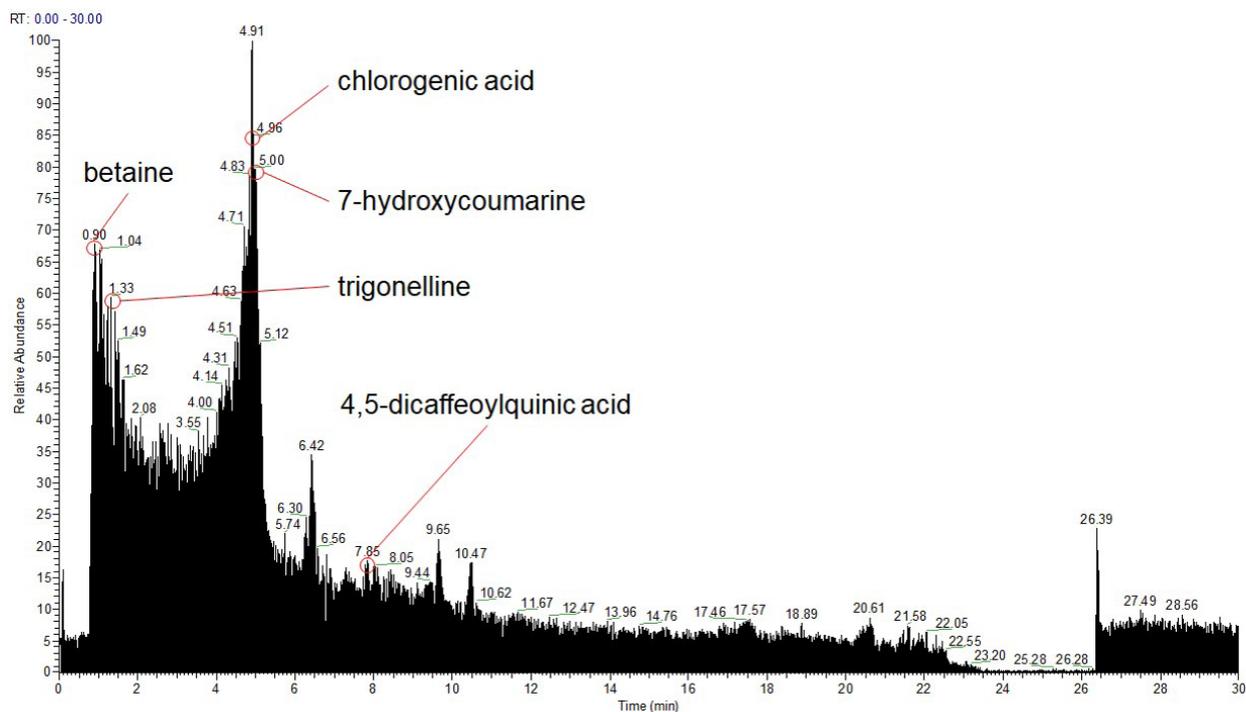
FIGURE 1. LC-HRMS chromatogram for Coriander extract by ethanol. Several compounds were detected such as betaine, trigonelline, chlorogenic acid, 7-hydroxycoumarine, and 4-5-dicaffeoylquinic acid which were known to have anti-diabetic activity
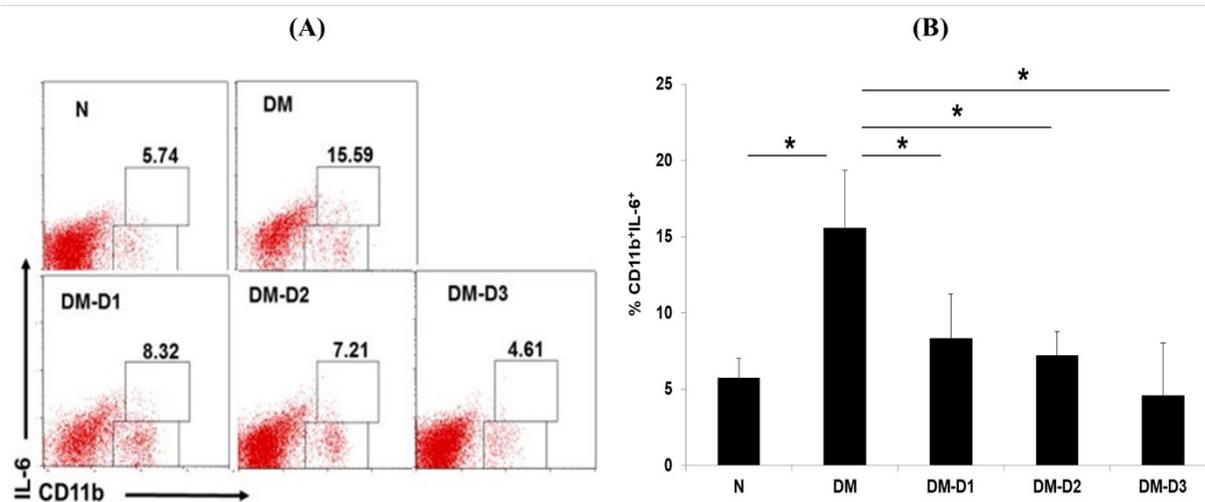

FIGURE 2. CSE suppressed IL-6 production in CD11b cells. (A). Diabetic mice were orally administered CSE for 2 weeks at different concentrations, as described in the methods section. Spleen cells (1×10^6) were obtained from the treated mice and subjected to cell surface staining with anti-CD11b followed by intracellular staining with anti-IL-6 antibody before being analysed by flow cytometry. Controls were normal healthy mice without induction. N, normal healthy mice; DM, diabetic mice; D1-3, doses of 25, 50 and 100 mg/kg BW. (B). Bars represent IL-6-expressing CD11b cells in mouse spleen cells receiving CSE. *$p < 0.05$, compared as indicated using one-way ANOVA (n=5)
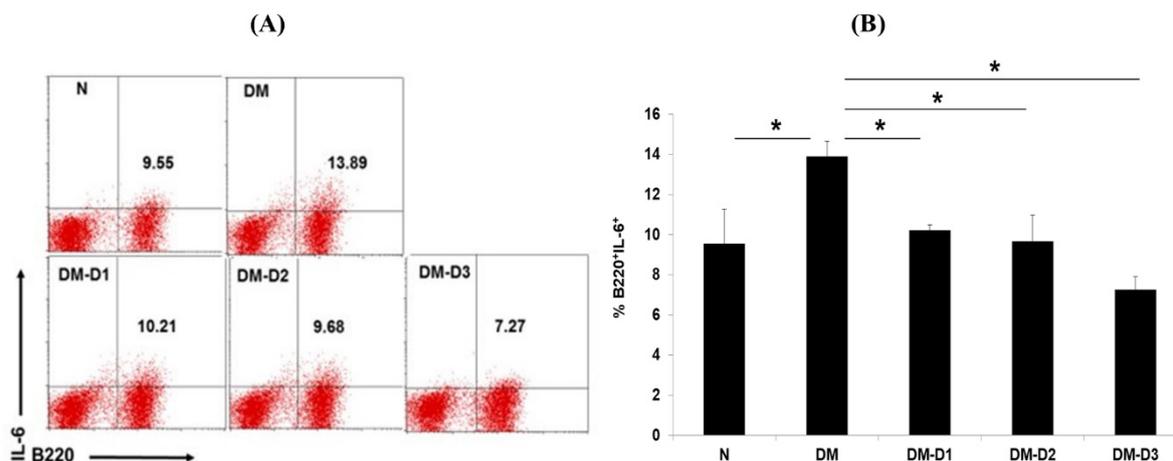
(A)

(B)

FIGURE 3. CSE suppressed IL-6 production in B220 cells. (A). Diabetic mice were orally administered CSE for 2 weeks at different concentrations, as described in the methods section. Spleen cells (1×10^6) were obtained from the treated mice and subjected to cell-surface staining with anti-B220 followed by intracellular staining with anti-IL-6 antibody before being analysed by flow cytometry. Controls were normal healthy mice without induction. N, normal healthy mice; DM, diabetic mice; D1-3, doses of 25, 50 and 100 mg/kg BW. (B). Bars represent IL-6-expressing B220 cells in mouse spleen cells receiving CSE. Data are mean ± SD of five mice in each group. *p < 0.05, compared as indicated using one-way ANOVA (n=5)

According to Arkatkar et al. (2017), the increase in IL-6 in B220 cells is a sign of B cell activation. Administering CSE is able to suppress the expression of IL-6 in B220 cells such that the expression reaches normal levels. In this study, we found evidence that administration of *C. sativum* to DM mouse models could suppress the synthesis of the pro-inflammation molecules IL-6. Polyphenols and flavonoids contained by *C. sativum* function as antioxidants allowed for a reduction of lipid oxides. These antioxidants can help overcome free radicals that are formed because of metabolism (Dhanapakiam et al. 2008; Rajeshwari et al. 2011; Sreelatha & Inbavalli 2012). IL-6 is a pro-inflammatory cytokine with pleiotropic properties that is important in diseases involving cell activation and causes insulin resistance and pathogenicity in the context of DM. In the pathogenicity of DM, inflammation plays an important role in the progression of the disease. Inflammation is often mediated by TNFα, IFNγ, IL-1B, IL-6 and IL-18. IL-6 is thought to have an immunoregulatory role by influencing glucose metabolism. IL-6 can act either directly or indirectly in the mechanism of disease progression. It indirectly affects muscle cells, pancreatic β-cells, adipocytes and neuroendocrine cells. Many factors affect cell response to IL-6 including the route of IL-6 administration and

cell type. The biological effects of IL-6 are numerous and complex, including differentiation, proliferation, survival and apoptosis. In DM mouse models, IL-6 was increased in both CD11b and B220 cells, indicating that cell activation occurs primarily in cells involved in the immune system. These data were consistent with the result that showed increased IL-6 in CD68 cells (Rifa'i et al. 2018).

DM patients experience an increase in pro-inflammation molecules such as TNFα, IFNγ, IL-1, and IL-6. In this study, we found evidence that CSE could inhibit the production of IL-6 in both CD11b and B220 cells. Production of IL-6 is increased in individuals who have DM and metabolic disorders (Esposito et al. 2002). IL-6 provokes insulin resistance and pathogenesis of type-2 DM by generating inflammation through the control of distinctions, migration, proliferation and cell apoptosis. Interestingly, IL-6 contributes to insulin resistance by degrading phosphorylation of insulin receptor and insulin receptor substrate-1 by inducing the expression of insulin signaling potential inhibitor, SOCS-3 (Rehman et al. 2017). Moreover, in DM mouse models, IL-6 was increased in CD11b and B220 cells, which in turn caused inflammation. CSE could inhibit the synthesis of IL-6 in both CD11b and B220 cells, but in this study, we were unable to explain by which

mechanism CSE blocked IL-6 synthesis. An increase of IL-6 in the DM mouse model was thought to trigger lipogenesis in the liver where B220 and CD11b cells could penetrate tissue and release IL-6, exacerbating existing inflammation. The high level of IL-6 in the DM mouse model was thought to be a result of an increase in ROS production (Rifa'i et al. 2018).

CSE PROMOTES CD62L EXPRESSION IN BOTH CD4 AND CD8 T CELLS

CD62L is also known as L-selectin, LAM-1 and Mel-14. It has a molecular weight of 74 to 94 kDa and is a transmembrane glycoprotein. CD62L is widely expressed in lymphocytes and can bind to glycoproteins that carry CD15, as well as to heparin sulfate. CD62L has a role in leukocyte rolling in the endothelium, as well as in preparing lymphocytes for homing in peripheral lymphoid tissue (Galkina et al. 2007). In this study, we observed a decreased expression of CD62L in DM mouse models (Figures 4 & 5). The loss of CD62L in lymphocytes indicated that lymphocytes were being activated. Accumulating evidence demonstrated that the activated CD4^{+} and CD8^{+} T cells contribute to obesity and insulin resistance known as the main critical risk factor for type 2DM (Xia et al. 2017). Coupled with that, the consequence of lymphocyte activation was an increase in the secretion of pro-inflammatory molecules such as IL-1, IL-6, TNF-α and IFN-γ, causing a worsening of the condition of the individual with type 2 DM. Dysregulated IL-6 can contribute to DM through several mechanisms including altered T cell trafficking (Hundhausen et al. 2016). From LC-HRMS analysis, we found that CSE contained an abundance of trigonelline (Figure 1). This compound had been confirmed by Subramanian et al. (2014) to function as antidiabetic and antidyslipidemic in high-fat-fed and low-dose streptozotocin-induced experimental diabetic rats. Trigonelline contained in CSE can ameliorate mouse health and result in a general decrease in the activation of pro-inflammatory molecules. Decreased cell activation is characterised by the appearance of CD62L molecules on the surface of CD4 and CD8 T cells.
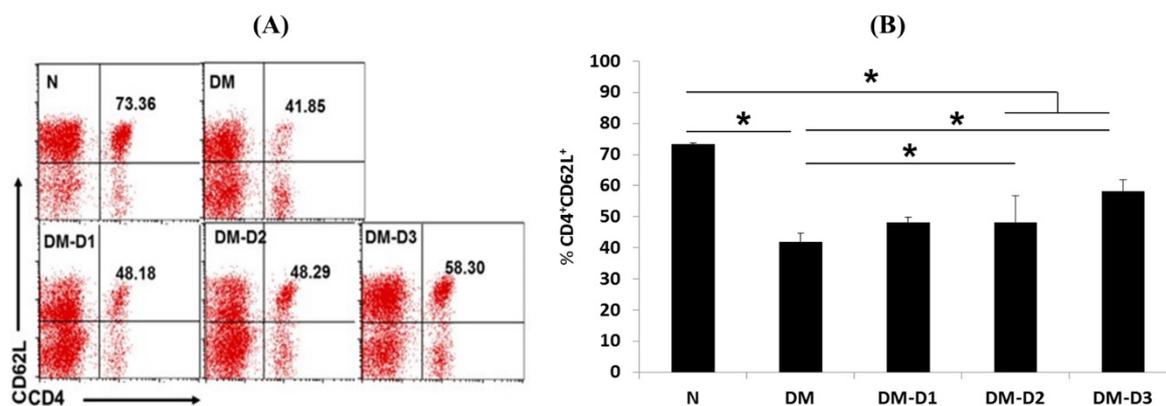

FIGURE 4. CSE increased CD62L molecule expression in CD4^{+} T cells (CD4^{+}CD62L(A). Diabetic mice were orally administered CSE for 2 weeks at different concentrations, as described in the methods section. Spleen cells (1×10^{6}) were obtained from the treated mice and subjected to cell surface staining with anti-CD4 and anti-CD62L antibodies before being analysed by flow cytometry. Controls were normal healthy mice without induction. N, normal healthy mice; DM, diabetic mice; D1-3, doses of 25, 50 and 100 mg/kg BW. (B). Bars represent CD62L-expressing CD4 T cells in mouse spleen cells receiving CSE. *p < 0.05, compared as indicated using one-way ANOVA (n=5)

In this study, we provide evidence that *C. sativum* increased CD62L molecular expression in both CD4 and CD8 T cells (Figures 4 & 5). A reduced proportion of CD62L^{+} molecules on the surface of the T cell indicated that the cells had been activated. Increased expression of CD62L molecules in peripheral lymphoid cells indicated a change in cell status from activated to naive type (Rabe et al. 2019). In this study, cells that had matured in the thymus when migrating to peripheral lymphoid tissue were not immediately activated but remained in a naive state for longer time. These data showed that DM mouse models that received *C. sativum*
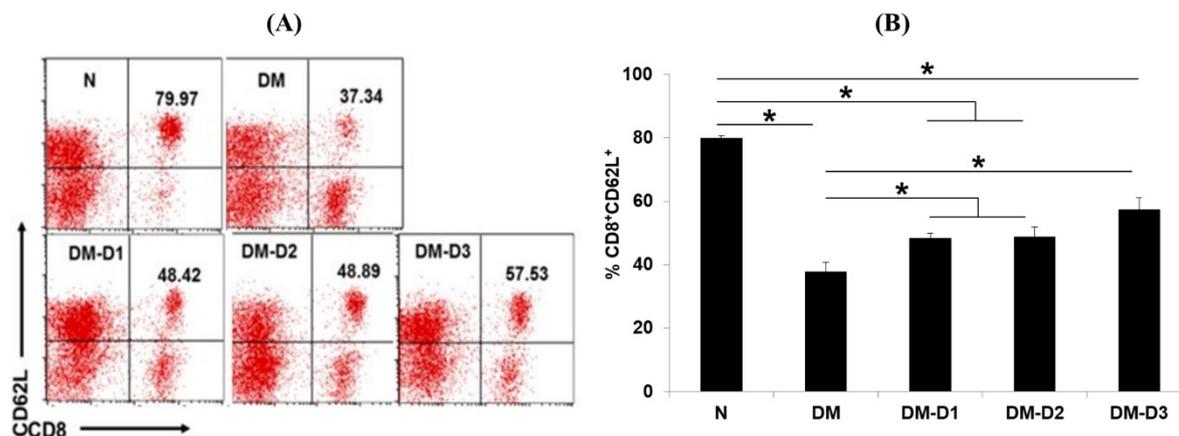
FIGURE 5. CSE increased CD62L molecule expression in CD8$^+$ T cells (CD8$^+$CD62L(A). Diabetic mice were orally administered CSE for 2 weeks at different concentrations, as described in the methods section. Spleen cells (1×10^6) were obtained from the treated mice and subjected to cell-surface staining with anti-CD8 and anti-CD62L antibodies before being analysed by flow cytometry. Controls were normal healthy mice without induction. N, normal healthy mice; DM, diabetic mice; D1-3, doses of 25, 50 and 100 mg/kg BW. (B). Bars represent CD62L-expressing CD8 T cells in mouse spleen cells receiving CSE. *p < 0.05, compared as indicated using one-way ANOVA (n=5)

improved their homeostasis such that most cells existed in a naive state. In DM mouse models, CD4 and CD8 T cells expressed CD62L molecules at a low level of 41 and 37%, respectively (Figures 4 & 5). Administration of *C. sativum* significantly increased the expression of CD62L molecules to 58% (p-value < 0.05). Increased expression of CD62L after administration of *C. sativum* indicated a physiological improvement in the DM mouse models such that excess activation of immunocompetent cells was prevented. The emergence of dominant CD62L molecules in CD4 and CD8 T cells indicated that general health in cells, tissues and organs was improved, thus inhibiting tissue damage. In this condition, we suspected that T cells were unable to find antigens that would otherwise trigger their activation. This fact was consistent with observations on B220 and CD11b cells that no longer synthesised large amounts of pro-inflammatory molecules IL-6 after being treated with *C. sativum* (Figures 2 & 3).

CSE INHIBITS CD25 EXPRESSION IN THE CD4 CELL POPULATION

CD25 is known as IL-2Rα and is often expressed by natural regulatory T cells. CD25 can also be expressed by activated cells in populations of T cells, B cells and monocytes. Resting cells express CD25 molecules at very low levels and are generally difficult to detect using flow cytometry or other techniques. The CD25 molecule is more involved in ligand binding than in signaling. The presence of CD25 on the cell surface can be used as a marker of cell activity due to an immune response. The CD25 molecule weighs 55 kDa and is a transmembrane glycoprotein that combines with CD122 and CD132 to form the intact IL-2 receptor (Kaku et al. 2017).

In this study, we obtained evidence that in the DM mouse model, the CD25 molecule increased in the CD4 T cell population (Figure 6). The increase in the CD25 molecule in DM mouse models indicated that there was more activated cell than increased regulatory T cells. In this experiment, cell activation accompanied a decrease in CD62L. Therefore, the increase in pro-inflammatory molecules also supported the idea that cells involved in the immune system undergo activation. On the other hand, the normality of homeostasis after administration of *C. sativum* may be due to the action of flavonoids, which play a role in improving pancreatic β cell proliferation, stimulating insulin secretion, reducing inflammation and maintaining blood glucose balance in adipose tissue. The administration of *C. sativum* to DM mouse models resulted in a shift in the proportion of cells expressing CD25 from high to normal levels. In conclusion, improvement of homeostasis occured in mice receiving oral CSE.
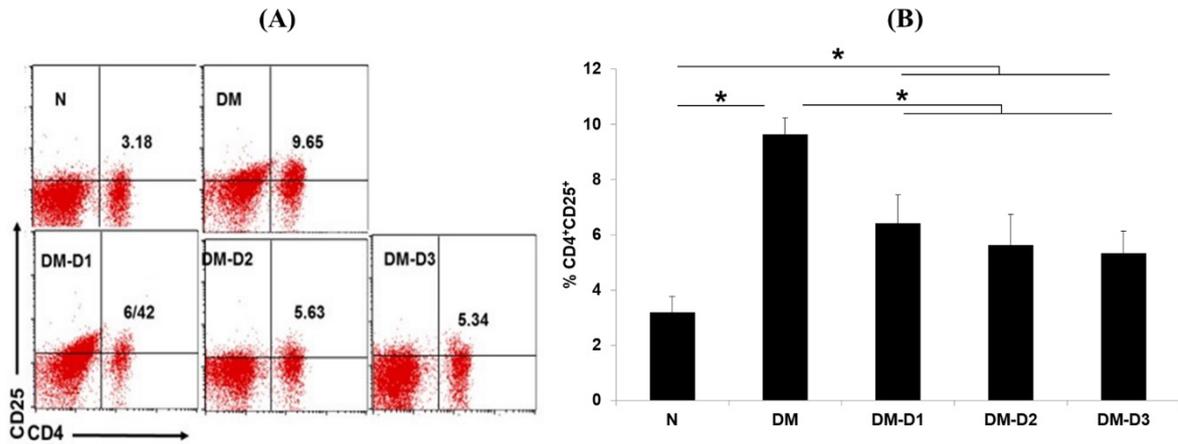
(A)

(B)

FIGURE 6. CSE inhibited development of CD4$^+$CD25$^+$ activated T cells. (A). Diabetic mice were orally administered CSE for 2 weeks at different concentrations, as described in the methods section. Spleen cells (1×10^6) were obtained from the treated mice and subjected to cell surface staining with anti-CD4 and anti-CD25 antibodies before being analysed by flow cytometry. Controls were normal healthy mice without induction. N, normal healthy mice; DM, diabetic mice; D1-3, doses of 25, 50 and 100 mg/kg BW. (B). Bars represent CD25-expressing CD4 T cells. *p < 0.05, compared as indicated using one-way ANOVA (n=5)

CSE DECREASES THE RATIO OF CD4 TO CD8 CELLS

CD4 T cells recognise antigen peptides that are complexed with class II major histocompatibility complex (MHC). The CD4 molecule is a transmembrane protein weighing 55 kDa expressed by helper T cells, monocytes, Langerhans cells, and dendritic cells. Another subset of T cell is cytotoxic CD8 lymphocytes. CD8 is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). Like the TCR, CD8 binds to an MHC molecule but is specific for the class I MHC protein. In normal individuals, the CD4:CD8 ratio is generally around two. If the CD4:CD8 cell ratio is less than one, it is considered that there is a disruption of homeostasis due to either infectious or genetic diseases. In this way, the CD4:CD8 cell ratio is a proxy for the health of an individual. In this study, we had evidence that the administration of *C. sativum* caused a reduction in the CD4:CD8 ratio (Figure 7). Administration of CSE triggered the development of CD8 T cells or suppressed the development of CD4 T cells. In normal mice, the CD4:CD8 ratio was around 2, while in the DM mouse model the ratio decreased to around 1. Administration of CSE reduced the ratio to 0.6 due to the predominance of CD8 T cells compared to CD4 cells. A large increase in CD8 T lymphocyte compensated for the decrease in CD4 T lymphocytes (Abuye et al. 2005).
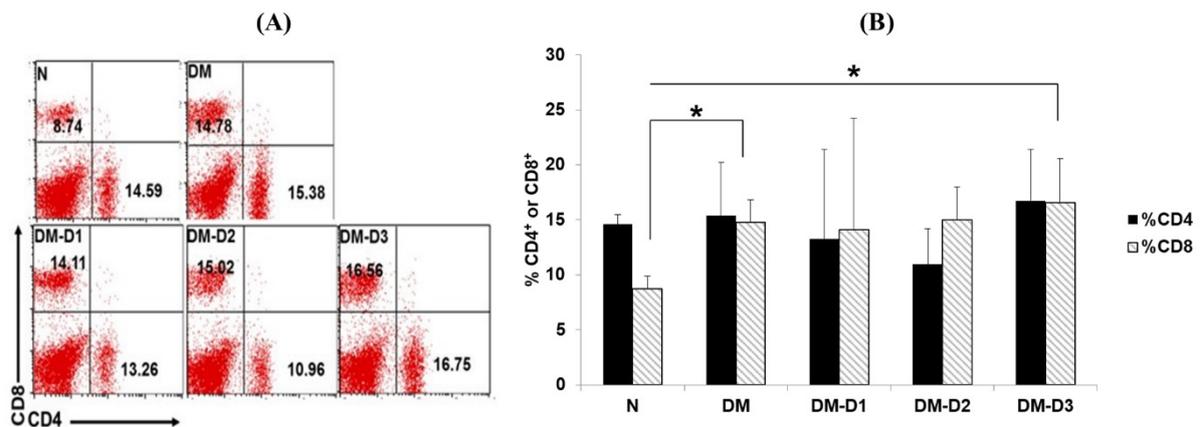
(A)

(B)

FIGURE 7. CSE decreased the ratio of CD4:CD8. (A). Diabetic mice were orally administered CSE for 2 weeks at different concentrations, as described in the methods section. Spleen cells (1×10^6) were obtained from the treated mice and subjected to cell surface staining with anti-CD4 and anti-CD8 antibodies before being analysed by flow cytometry. Controls were normal healthy mice without manipulation. N, normal healthy mice; DM, diabetic mice; D1-3, doses of 25, 50 and 100 mg/kg BW. (B). Bars represent the relative number of CD4 and CD8 T cells in spleen cells receiving CSE. *p < 0.05, compared as indicated using one-way ANOVA (n=5)

In this study, we were unable to explain with certainty why CD8 became dominant after CSE administration (Figure 7). However, we hypothesised that the reduction in CD4 T cells is a physiological response to avoid increased inflammation, since CD4 T cells have an enormous potential to encourage the development of effector cells (Ahrends et al. 2017). CD4 T cells have important products, such as IL-2, which influences the proliferation of effector. The decrease in CD4 T cell number is a physiological strategy to reduce inflammation, while the increase of CD8 T cells can be considered a compensatory effect.

CONCLUSION

In this study, we found that DM model mice displayed an increase in pro-inflammatory IL-6 molecules. The administration of CSE resulted in pro-inflammatory molecule suppression in both B220 cell and CD11b cell populations. Decreased expression of IL-6 was in line with the inhibition of T lymphocyte cell activation in both CD4 and CD8 cell populations. Following the administration of *C. sativum*, $CD4^+CD62L^+$ and $CD8^+CD62L^+$ naive T cells showed increased development. In this same experiment, we provided evidence that the population of $CD4^+CD25^+$ cells increased in DM model mice and that the administration of *C. sativum* controlled $CD4^+CD25^+$ number to achieve a normal physiology. The ratio of CD4:CD8 in this study decreased after the administration of *C. sativum*. The domination of CD8 cells compared to CD4 cells after the administration of *C. sativum* was synergistic with the inhibition of effector cell development (both $CD4^+CD62L^-$ and $CD8^+CD62L^-$).

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