Immunomodulatory Effects of Formulation of *Channa micropeltes* and *Moringa oleifera* through Anti-Inflammatory Cytokines Regulation in Type 1 Diabetic Mice

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**Abstract**

**Background:** Diabetes mellitus type 1 (T1DM) is an autoimmune disease characterized by chronic inflammation of the \(\beta\)-pancreas cells. The immunomodulatory activities related to the role of anti-inflammatory cytokines might contribute to control the inflammatory response in T1DM. This study aimed to evaluate the immunomodulatory effect of *Moringa oleifera-Channa micropeltes* formulation (MC) based on the profile of CD4\(^+\)IL-4\(^+\), CD4\(^+\)IL-10\(^+\), and CD4\(^+\)TGF-\(\beta\)\(^+\) in type 1 diabetic mice.

**Methods:** A total of 30 mice were divided into six equal groups as normal, diabetic mice, diabetic mice with metformin administration, and diabetic mice with MC administration doses 1, 2, and 3. The mice were injected intraperitoneally by 145 mg/kg BW Streptozotocin (STZ) to induce T1DM. Diabetic mice were orally administrated by MC for 14 days. The levels of CD4\(^+\)IL-4\(^+\), CD4\(^+\)IL-10\(^+\), and CD4\(^+\)TGF-\(\beta\)\(^+\) were determined by flow cytometry analysis.

**Results:** The DM group had low levels of IL-4 and IL-10, but high levels of TGF-\(\beta\) as compared to the normal. Administration of MC in certain doses significantly increased the levels of IL-4 and IL-10, while inversely decreased the levels of TGF-\(\beta\) in diabetic mice at the levels close to the normal and significantly different from the DM group. The glucose levels in diabetic mice after MC administration were significantly lower than the DM group.

**Conclusion:** Based on the results, MC administration in a dosage-dependent manner might have the immunomodulatory effect to reduce the inflammation by increasing IL-4 and IL-10 and suppressing TGF-\(\beta\) in type 1 diabetic mice.

**Introduction**

Diabetes mellitus (DM) is a metabolic disorder characterized by increased blood sugar levels (hyperglycemia).\(^{1}\) DM globally affects 463 million people in 2019 and will predictably rise to 700 million in 2045.\(^{2}\) Approximately 5-10% of DM patients are having type 1 DM (T1DM).\(^{3}\) T1DM patient is characterized by the destruction of insulin-producing \(\beta\) pancreas islet cell. The main cause of \(\beta\)-pancreas destruction in T1DM is an autoimmune response which is characterized by a chronic inflammation.\(^{4}\) Hyperglycemia induces the production of reactive oxygen species (ROS) which can activate the transcription factor for pro-inflammatory cytokines,\(^{5}\) thereby inducing the inflammation. The pro-inflammatory cytokines are highly expressed in T1DM\(^{6}\) and enhance \(\beta\)-cells destruction by exacerbating immune cell infiltration to attack and induce apoptosis of \(\beta\) cells.\(^{7}\) The development of chronic inflammation in T1DM may occurs due to the impairment of immunomodulation in the immune system to control the inflammation. Immunomodulation plays an important role in maintaining homeostasis of the immune system by modulating some activities such as stimulation, suppression, and restoration of the immune response.\(^{8}\) One of the immune components involved in the activities of immunomodulation is anti-inflammatory cytokines due to their suppressive activity. Anti-inflammatory cytokines can control the inflammatory response by repressing the expression of pro-inflammatory cytokines.\(^{9}\) Some cytokines that have been known for anti-inflammatory properties and implicated in \(\beta\) cell viability during T1DM are interleukin (IL)-4, IL-10, and tumor growth factor-beta (TGF-\(\beta\)).\(^{9,10}\) Based on its function, we assume that these cytokines might offer therapeutic potential in T1DM which is associated with an inflammation-induced autoimmune attack.

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DM is treated by insulin and hypoglycemic drug to control the blood glucose. The use of hypoglycemic drug like metformin have side effects in the patients because long term consumption of this drug can damage renal and liver organs. Hence, an alternative treatment from natural resources is needed to minimize the side effects. Some of these natural resources are *Moringa oleifera* (MO) leaves and *Channa micropeltes* (CM). In this study, we used the formulation of MO and CM (MC).

MO has been well known for anti-inflammatory, antioxidant, and anti-diabetic activities because of its nutrition. MO leaves contain vitamins, minerals, and rich in polyphenols such as flavonoid, phenolic acid, tannins, etc. The anti-inflammatory effect of MO is by inhibiting gene expression of pro-inflammatory cytokines. CM has a local name toman fish and comes from Kalimantan Island in Indonesia. It contains albumin protein, omega-3, omega-6, and minerals. The previous study showed that albumin obtained from another species of *Channa*, haruan fish (*Channa striata*), could inhibit the production of inflammation mediators in diabetic conditions. Albumin levels in CM are known higher than haruan fish. We assumed that the potential role of this formulation due to the nutrition value in MO and CM that had anti-inflammatory activities so both could work in synergy. Based on that, we hypothesized that MC would be more effective in producing an immunomodulatory effect in controlling the inflammatory response in T1DM, especially in regulating anti-inflammatory cytokines. Furthermore, the potential role of anti-inflammatory cytokines in the prevention of T1DM progression was less studied. Therefore, this study aimed to evaluate levels of IL-4, IL-10, and TGF-β after administration of MC in mice model of T1DM.

**Materials and Methods**

**Extract preparation and formulation of Moringa oleifera-Channa micropeltes**

MO leaves were extracted using water according to Engsusan and El-Gammal with modification. The extraction of MO leaves was performed by dissolving 5 grams leaves powder of MO (Materia Medica Batu, Malang, Indonesia) in 50 mL of boiled water. The extract solution was filtered with Whatman no. 1 paper and the water was removed using a freeze dryer. The MO extract was combined with CM extract to prepare 3 MC doses. The CM extract was obtained from Ifalmin® product (PT. Ismut Fitomedika, Makassar, Indonesia). Ifalmin® is a supplement product which contained of CM flesh extract. The extraction of CM was initiated by trimming the fins and disposing of scales. The gills and offal were disposed to obtain the flesh for extraction. The extraction of CM flesh was performed at a temperature of 50 °C using water as a solvent and the extract was dried up using a freeze dryer. Doses formulation was determined based on the effective dose of MO leaves for DM treatment and the recommended dose of Ifalmin® consumption from the manufacture. The effective and safe dose of MO leaves from another studies was 800 mg/kg BW. The recommended dose of Ifalmin® was 615 mg/kg BW after conversion of human dose to animal equivalent dose (AED) according to food and drug administration (FDA). These doses were used in the formulation of MO and CM with modification as follow: D1 (MO 800 mg/kg BW: CM 800 mg/kg BW), D2 (MO 615 mg/kg BW: CM 615 mg/kg BW) and D3 (MO 800 mg/kg BW: CM 615 mg/kg BW).

**Animal and type 1 diabetes induction**

Male mice (*Mus musculus*), strain BALB/c, with the ages of 8-10 weeks were acclimatized for seven days in the free-pathogen room at Animal Anatomy and Physiology Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia. The mice were obtained from Singsosari Breeder (Malang, Indonesia). The study procedures were approved by The Ethical Committee of Brawijaya University (Reg. No. 1180-KEP-UB).

The induction of T1DM was performed using a single high dose of Streptozotocin (STZ). STZ at a dose of 145 mg/kg BW was injected intraperitoneally once to obtain a mice model of T1DM. Mice fasted for 4 hours before STZ injection. Blood glucose was measured using Easy Touch glucometer (Bioptik Technology Inc., Taiwan) four days after STZ injection. The mice were considered diabetic when they had a glucose level exceed 200 mg/dL.

**Experimental design and treatment**

A total of 30 mice were divided into six groups. There were 25 diabetic mice randomly divided into five equal groups as diabetic mice (DM), diabetic mice with metformin administration (DM-M), and diabetic mice with MC administration dose 1 (DM-D1), dose 2 (DM-D2), and dose 3 (DM-D3). Moreover, five non-diabetic mice allocated as normal groups (N).

MC was administrated orally for 14 days in type 1 diabetic mice (DM-D1, DM-D2, and DM-D3). The blood glucose levels of experimental mice were measured once in three days for 15 days. In this study, we used metformin as a standard drug in DM treatment. Metformin (PT. Hexparm Jaya, Bekasi) at a dose of 307.5 mg/kg BW mice was dissolved in water. Metformin was orally administrated in the DM-M group treatment. The conversion of human dose to AED in this research is determined according to FDA.

**Immunostaining and flow cytometry**

Antibody staining was performed according to Rifai and Widodo to determine the levels of IL-10, IL-4, and TGF-β. On day 15, the mice were sacrificed by cervical dislocation technique without using carbon dioxide or others chemistry agents after measurement of blood glucose levels for the last time. The cervical dislocation was initiated by restraining the mice on the flat surface in the normal standing position, then the base tail of mice.

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was firmly grasped by one hand. The closed scissor was placed against the back of the mice's neck at the base of the skull. The dislocation was performed by pushing forward the scissor which restrained the head while pulling back the tail by hand. Then, the spleen organ was isolated from the mice to obtain the cytokine-expressing lymphocyte cell. The spleen organ was homogenized in Phosphate Buffer Saline (PBS) in the petri dish with a plunger of 10 mL syringe. The homogenate was moved into microtube and centrifuged at 2500 rpm, 10 °C for 5 minutes. The supernatant was removed, and the pellet was moved into a microtube containing 300 µL-400 µL of PBS for antibody staining, then the sample was centrifuged.

The antibodies based on the protocol's company were applied at a concentration of 0.005 mg/100 µL. The cells were extracellularly stained with 50 µL of FITC-conjugated rat anti-mouse CD4 (Biolegend®, San Diego) before intracellular staining and incubated for 20 minutes in the icebox (4 °C). Intracellular cytokine staining was initiated by adding the cells with 50 µL Cytofix (BD-Biosciences Pharmingen) and incubated for 20 minutes in the icebox. Then, the cells were added with 400-500 µL Wash Perm Solution (WPS) (BioLegend®, USA) and centrifuged at 2,500 rpm, 10 °C for 5 minutes. The supernatant was removed from the sample and each pellet was stained with 50 µL of PerCP-conjugated rat anti-mouse IL-10 (Biolegend®, San Diego), PE/Cy5-conjugated rat anti-mouse IL-4 (Biolegend, San Diego), and PE/Cy5-conjugated rat anti-mouse TGF-β (Biolegend, San Diego). The sample was placed into a flow cytometry tube and added with 400 µL of PBS. Flow cytometry tube was put into the flow cytometer (BD FACSCalibur, USA) to count the levels of target cytokines.

Data analysis

Data from flow cytometry were analyzed using BD Cellquest Pro™ software (BD Biosciences, San Jose, CA, USA). The data were then statistically analyzed using the SPSS program with one way of variance analysis (ANOVA) and the p-value < 0.05.

Results

MC treatment reduced glucose levels in type 1 diabetic mice

The initial levels of glucose in diabetic mice (DM, DM-D1, DM-D2, DM-D3, and DM-M) ranged above 300 mg/dL and significantly higher than the normal mice (p<0.05) (Figure 1). The levels of blood glucose in normal mice were consistently lower than in other groups for 15 days. The administration of MC for 14 days gave the anti-hyperglycemic effect in diabetic mice that were showed by decreased glucose levels from the initial levels. The glucose levels in DM-D1 and DM-D2 groups were not significantly different as compared to the normal group on day 15th. The glucose levels in DM-D3 and DM-M groups on day 15th were significantly higher than the normal group and not significantly different from the DM-D1 and DM-D2 groups. However, the glucose levels in diabetic mice with administration of metformin (DM-M) and MC (DM-D1, DM-D2, DM-D3) were significantly different as compared to the DM group (p<0.05). The glucose levels in diabetic mice after administration of MC for 14 days was 222 mg/
dL (DM-D1), 265 mg/dL (DM-D2), and 291 mg/dL (DM-D3) respectively meanwhile the glucose levels in the DM group without MC administration increased continuously to 496 mg/dL until the last day of measurement (Figure 1). The final levels of glucose in all MC treatment groups were significantly lower than the DM group even though the values had not reached normal levels.

**MC treatment increased IL-10 in type 1 diabetic mice**
The levels of CD4⁺IL10⁺ in the DM group were significantly lower than the normal group (p<0.05) (Figure 2). The levels of IL-10 in the DM-M group were 11.45% and not significantly different from the normal group. IL-10 levels in the DM-D1 group (6.49%) were not significantly different from the DM group. Administration of MC with doses 2 and 3 in diabetic mice significantly increased the levels of IL-10 to 9.66% and 8.47% respectively, compared to the DM group (p<0.05). IL-10 levels in the DM-D2 and DM-D3 groups were not significantly different from the normal group. This indicated that the administration of MC based on a dose-dependent was able to restore the decreased levels of IL-10 in diabetic mice to the normal levels.

**Figure 2.** MC administration increased the levels of IL-10 expressed by CD4⁺-expressing cells in type 1 diabetic mice. (A) Spleen cells (2x10⁶) were obtained from all mice, then subjected to extracellular staining cells with anti-CD4 antibody, intracellular staining cells with anti-IL-10 antibody, and analyzed by flow cytometry. N: non-diabetic mice without STZ induction; DM: type 1 diabetic mice with STZ induction without MC treatment; DM-D1: type 1 diabetic mice with MC treatment dose 1 (MO 800 mg/kg BW: CM 800 mg/kg BW); DM-D2: type 1 diabetic mice with MC treatment dose 2 (MO 615 mg/kg BW: CM 615 mg/kg BW); DM-D3: type 1 diabetic mice with MC treatment dose 3 (MO 800 mg/kg BW: CM 615 mg/kg BW); DM-M: type 1 diabetic mice with metformin treatment. (B) The bars are a calculation of CD4⁺IL10⁺ in splenic cells. The data are mean value ± SD of five mice in each group with a significant value p<0.05 (n = 30).

**Figure 3.** MC administration increased the levels of IL-4 expressed by CD4⁺-expressing cells in type 1 diabetic mice. (A) Spleen cells (2x10⁶) were obtained from all mice, then subjected to extracellular staining cells with anti-CD4 antibody, intracellular staining cells with anti-IL-4 antibody, and analyzed by flow cytometry. N: non-diabetic mice without STZ induction; DM: type 1 diabetic mice with STZ induction without MC treatment; DM-D1: type 1 diabetic mice with MC treatment dose 1 (MO 800 mg/kg BW: CM 800 mg/kg BW); DM-D2: type 1 diabetic mice with MC treatment dose 2 (MO 615 mg/kg BW: CM 615 mg/kg BW); DM-D3: type 1 diabetic mice with MC treatment dose 3 (MO 800 mg/kg BW: CM 615 mg/kg BW); DM-M: type 1 diabetic mice with metformin treatment. (B) The bars are a calculation of CD4⁺IL-4⁺ in splenic cells. The data are mean value ± SD of five mice in each group with a significant value p<0.05 (n = 30).
**MC treatment increased IL-4 in type 1 diabetic mice**

T1DM condition significantly decreased the levels of CD4^+IL-4^+ from 11.18% to 5.25% compared to the normal group (p<0.05) (Figure 3). Administration of metformin significantly increased IL-4 levels to 19.4% compared to the DM group, but the levels were higher than the normal which was marked by a significant difference in IL-4 levels between the normal and DM-M groups (p<0.05). Administration of MC with doses of 1, 2, and 3 was significantly able to increase the levels of IL-4 approaching normal level compared to the DM group (p<0.05). The levels of IL-4 in DM-D1, DM-D2, and DM-D3 were 10.82%, 7.66%, and 7.45%, but only the DM-D1 group, which was not significantly different from the normal group. This indicated that dose 1 was an effective dose to restore the levels of IL-4 to normal levels in type 1 diabetic mice after MC treatment for 14 days.

**MC treatment decreased TGF-β in type 1 diabetic mice**

The levels of TGF-β secreted by CD4^+ T cell in the normal group was 7.4%, then it increased significantly to 10.65% in the DM group (p<0.05) (Figure 4). TGF-β levels in the DM-M group (11.29%) were not different from the DM group. Administration of MC with doses 1 and 3 in diabetic mice was significantly able to decrease the levels of TGF-β to 8.65% and 6.91% compared to the DM group (p<0.05). The TGF-β levels in DM-D1 and DM-D3 groups were not significantly different from the normal group. These results showed that the administration of MC with doses 1 and 3 could restore the increased levels of TGF-β approaching normal levels in the type 1 diabetic mice.

**Discussion**

The autoimmune response in T1DM occurs due to the loss of immunologic tolerance which results in infiltration of immune cell to destroy insulin-producing β-pancreas cells. The infiltrating immune cells secrete pro-inflammatory cytokines which facilitate the dysfunction and damage of β-cells. Anti-inflammatory cytokines play an important role in diminishing the inflammatory response in inflammatory diseases, including T1DM, due to their suppressive. This study proved that the levels of anti-inflammatory cytokines IL-10 and IL-4 were significantly reduced in mice model of T1DM as compared to the normal (Figures 2 and 3). This showed that decreasing levels of anti-inflammatory IL-10 and IL-4 might contribute to the progression of the inflammatory response during T1DM. The decline of IL-10 and IL-4 levels contributes to the inhibition of immune-regulatory activities in the regulation of inflammatory response and prevention of autoimmunity.

The high levels of TGF-β in the DM group as compared to the normal group (Figure 4) were assumed due to the hyperglycemia condition and high production of ROS after STZ induction. STZ can increase the levels of ROS. This study proved that STZ induced the T1DM which was marked by hyperglycemia (Figure 1). Hyperglycemia in T1DM as well increases ROS production and causes the imbalance state between ROS and antioxidant enzymes, thereby inducing oxidative stress.

The suppression activities of each anti-inflammatory cytokine have distinct mechanisms. IL-4 inhibits expression of pro-inflammatory interferon-gamma (IFN-γ) and activity of pro-inflammatory IL-1β by blocking the binding with its receptors. IL-10 exerts anti-inflammatory effect by...
suppressing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor for pro-inflammatory cytokines, through inhibition of I kappa-B kinase (IkK) activity and block of NFκB DNA binding.24 Suppressive activities of TGF-β occurs through inhibition of Th1 differentiation by reducing IL-12 receptor and stimulation of inducible regulatory T cell (iTreg) generation by inducing Foxp3 expression.35 Activities of these suppressive cytokines may potentially contribute to preventing the inflammatory response in T1DM. Meanwhile, TGF-β as an anti-inflammatory cytokine seems to play less role in T1DM but conversely promotes the progression of this disease wherein correlates to the raise of TGF-β levels in type 1 diabetic mice from the present study. The detrimental effect of TGF-β in pancreatic β-cell can be a consequence of its pleiotropic effect. Activation of TGF-β signaling declined the replication of pancreatic β cells through Ink4a expression.36 High levels of TGF-β were related to the progression of DM complications in T1DM43 and possibly contributed to pathological changes in the pancreas.44 TGF-β also exerts pro-inflammatory effect by promoting the differentiation of Th17 cells in the presence of IL-6 through transcription factor IκBζ,45 so this can worsen the inflammatory response in T1DM.

The destructive effect of the inflammatory response on β-pancreatic cells can be overcome through the activation of suppressive cytokines IL-10 and IL-4 after the administration of MC in type 1 diabetic mice. IL-10 and IL-4 secreted by Th2 cells can prevent the islet inflammation which causes β-cell destruction and the secretion of these cytokines correlates with T1DM protection.46 Furthermore, anti-inflammatory cytokines possibly promote the cytoprotection effect on β-pancreatic by elevating the expression of anti-apoptotic genes.9 MC with certain doses increased the levels of IL-10, IL-4 and decreased TGF-β levels in type 1 diabetic mice at the levels which were not significantly different from the normal mice (Figure 2-4). The bioactive compounds of MO that possibly modulates the expression of anti-inflammatory cytokine are quercetin and vitamin A. Quercetin has an anti-inflammatory effect by inducing the expression of IL-10.31 Quercetin is also reported to inhibit the expression of TGF-β.42 MO leaves contain high levels of β carotene, a precursor of vitamin A.13 Administration of vitamin A increased the gene expression of IL-4 in another inflammatory disease.43 Furthermore, MO contains high levels of flavonoid which exerts high antioxidant activity by donating its one electron to some radicals.44 Antioxidant is often used to treat inflammatory diseases like DM which is follow by oxidative stress.

CM has high levels of albumin which serves as an exogenous antioxidant. The immunomodulatory effect of albumin in CM to anti-inflammatory cytokines possibly occurs through an antioxidant mechanism. Albumin is known able to activate the signaling of nuclear factor erythroid2-related (Nrf2) for antioxidant and other enzymes, including heme oxygenase-1 (HO-1).45 Activation of HO-1 eventually promotes the anti-inflammatory activity by inducing the expression of anti-inflammatory cytokines IL-10 and IL-4.46 Albumin possibly suppressed the production of TGF-β in type 1 diabetic mice through ROS levels reduction. Albumin has abundant sulphhydril (-SH) groups which can bind with free radical so that it can decrease the levels of ROS.45 We also compared the effect of MC in anti-inflammatory cytokines with a standard drug, metformin. Metformin exerted its anti-inflammatory effect by up-regulating the expression of anti-inflammatory cytokines IL-10 and IL-4.48 The increase of IL-10 and IL-4 in type 1 diabetic mice after metformin treatment was also seen in this study even the levels were significantly higher than the normal mice (Figure 2 and 3). Metformin could reduce the levels of TGF-β in diabetic patients,49 which was contrary to the raise of TGF-β levels in this study after metformin administration. The dose of metformin used in this study might not be enough to reduce the levels of TGF-β. Interestingly, the levels of IL-10 at a dose 1 and the levels of TGF-β at a dose 2 was not significantly different as compared to the the DM group. Meanwhile, the levels of IL-4 at doses 2 and 3 were significantly different than the normal and DM-D1 eventhough the levels were significantly higher than the DM group. Based on it, we assumed that the ability of MC to increase IL-10 and IL-4 levels or decrease TGF-β levels in type 1 diabetic mice was possibly affected by the concentration of CM and MO which varied in each dose formulation. Furthermore, it needs further studies with more parameters to confirm the mechanism how MC works as this research is a preliminary study with limited parameters. However, this study revealed that MC potentially had immunomodulatory effects which were showed by its abilities to restore the high or low levels of IL-4, IL-10, and TGF-β in type 1 diabetic mice. Immunomodulator has a role to stimulate, suppress or restore the immune response.2

The abilities of MC in increasing the levels of IL-10 and IL-4 while decreasing the levels of TGF-β represents its immunomodulatory activities in T1DM to reduce the inflammation and may impact the amelioration of β-pancreas cells. Reduction of inflammation played by anti-inflammatory cytokines function to limit the tissue damage and maintain the tissue homeostasis,50 thereby contributing to β-pancreas reparation. Then, the insulin can be secreted normally by β-pancreas cells. The efficacy of MC to diminish the progression of T1DM was in line with a decrease of blood glucose levels from the initial levels closer to normal levels < 200 mg/dL (Figure 1).

Conclusion

Based on the results, it can be concluded that Moringa oleifera-Channa micropeltes formulation in a dosage-dependent manner might have the immunomodulatory effect to reduce the inflammatory response in the experimental mice model of T1DM through the increase of
Immunomodulatory effect of Channa micropeltes-Moringa oleifera in T1DM mice

IL-4 and IL-10 and suppression of TGF-β. This formulation can be used as adjuvant therapy in the early stages of T1DM during the active inflammation, and further studies with a large sample size are needed to verify and confirm the results.

Ethical Issues
All the procedures using the animal model in this research have been reviewed and approved by Ethical Committee of Brawijaya University (Reg. No. 1180-KEP-UB).

Conflict of Interests
The authors claim that there is no conflict of interest.

References


