Effect of pegagan (*Centella asiatica*) nanoparticle coated with chitosan on the cytokine profile of chronic diabetic mice

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Abstract

Diabetes is closely related to immune response problems when it occurs chronically. Pegagan (*Centella asiatica*) is a medicinal plant with active compounds. Madecassoside is beneficial in treating diabetes, and nanoparticle technology is expected to enhance the medicinal potential and availability of pegagan compounds. The aim of this study was to determine the effect of chitosan-coated pegagan nanoparticles on the cytokine profile of chronic diabetic mice, which included CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+, CD8+IFN-γ+ and IL-6+. An experimental study with a randomized complete block design (CRD) consisting of six treatments with seven replicates was conducted. The groups were: healthy mice as negative control; diabetic mice treated with distilled water as positive control and diabetic mice treated with nanoparticle coated with chitosan (NPC) 20 mg/kg, 30 mg/kg, 40 mg/kg, and metformin 130 mg/kgBW. The data were tested using one-way analysis of variance (ANOVA) with a significance level of 5% and continued with the Duncan's multiple range test. The results showed that pegagan NPC could significantly reduce the relative number of CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+ and CD8+IFN-γ+ and IL-6 in the dose of 20 mg/kg, 30 mg/kg and 40 mg/kg (*p*<0.05). The treatment dose of 20 mg/kg reduced CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+, CD8+IFN-γ+ to the levels of healthy mice and a dose of 30 mg/kg could reduce IL-6 as in healthy mice. These findings suggest that chitosan-coated pegagan nanoparticles are a promising therapy for diabetes, as they have the potential to modulate the immune response associated with chronic diabetes.

Keywords: Diabetes mellitus, immune system, antidiabetic, *Centella asiatica*, nanomedicine

Introduction

Diabetes is a metabolic disorder characterized by a failure to metabolize glucose, resulting in high blood sugar concentrations [1-3]. Chronic diabetes can be characterized by hyperglycemia that occurs for a long time without treatment, which can cause pathological and functional changes that trigger macrovascular and microvascular complications [4]. The pathogenesis of diabetes and the development of vascular disorders in chronic diabetes are closely related to problems with the body's immune response [5]. The main elements involved in adaptive

immunity are antibodies, B lymphocytes, and T lymphocytes [6,7]. T cells are categorized into two main groups based on the class of MHC molecules recognized by the T cell receptor (TCR) [6]. Activated CD4 T cells can generate strong inflammation by producing proinflammatory cytokines. When CD4 T cells are activated in the presence of interleukin 12 (IL-12), they become Th1 phenotype secreting interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) into the environment, inducing inflammation [8]. CD8 T cells perform an effector function, which is to defend against infected host cells [6]. Several subsets of CD8 T cells have been identified, including Tc1, which produces IFN-γ and TNF-α [9]. TNF-α, IL-6 and the IFN family, such as IFN-γ, are cytokines that promote the inflammatory cascade and are considered proinflammatory mediators [10]. Cytokines are responsible for the activity, differentiation, proliferation and production of immune cells or other cytokines and play a role in attracting immune cells and signaling molecules to the site of inflammation [11].

A previous study indicated that in diabetes with chronic hyperglycemic conditions, there is an increase in pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 [12]. Another study revealed an increase in TNF-α, IL-6 and IFN-β in neutrophils of patients with diabetic complications when compared to normal people [5]. Patients with diabetic nephropathy also showed an increase in serum Th1 and IFN-γ [13]. Diabetes not only affects the activity of pro-inflammatory cytokines but also increases pro-inflammatory T cells, which can cause tissue and systemic inflammation [14]. The adaptive immune system, in particular T lymphocytes, also plays an important role in the pathogenesis of diabetes [15].

A previous study found that there was penetration of CD4+ and CD8+T cells into the renal interstitial tissue in the streptozotocin (STZ)-induced diabetic mouse model [16]. In addition, IFN-γ and TNF-α levels also increased significantly in the kidneys of diabetic mice compared to control mice [16]. It is known that the number of CD8 also increased in diet-induced obese mice, and the accumulation of CD8 can induce inflammation and insulin resistance [14]. The imbalance of inflammatory mediators in diabetics can cause and trigger the development of complications in diabetes; therefore, there is a need for immunomodulators, which are substances that can regulate the imbalance of the disturbed immune system [17,18].

Immunomodulation serves to modulate immunological parameters that control disease using immunomodulators [19]. The use of various plant extracts and their secondary metabolites as immunomodulators shows a broad spectrum of anti-inflammatory and immunomodulatory activities with relatively few safety concerns, and one of the plants used is pegagan (*Centella asiatica*) [20]. Pegagan has benefits that have been widely assessed before, including as an antimicrobial [21], anti-inflammatory [22], antidiabetic [23], antihyperglycemic [24], and has good antioxidant activity [25]. It contains the main active compounds that belong to the class of triterpene compounds, such as asiatic acid, asiaticoside, and madecassoside. Pegagan also contains flavonoid compounds such as quercetin and kaempferol [26]. A previous study revealed that flavonoid compounds could act as an anti-inflammatory by blocking the secretion of IL-1, IL-6, IFN-γ, and TNF-α in the blood induced by *Escherichia coli* lipopolysaccharide [27]. Another study revealed that pegagan methanol extract given for 14 days to type 2 diabetes model mice significantly reduced pro-inflammatory mediators such as TNF-α and monocyte chemoattractant protein-1 (MCP-1) in the liver when compared to untreated diabetic mice [28].

In the utilization of pegagan as a medicinal plant, it is necessary to have a method that could maximize its potential. The utilization of pegagan is mostly in the form of extracts [28,29,30]. However, the extract form still has shortcomings, such as the particle size being too large and the crude extract having low-fat solubility, permeability, and bioavailability [22]. It is expected that processing into nanoparticles could increase the potential of pegagan as a medicinal plant because the size of nanoparticles could make it easier to penetrate the intercellular spaces, and increase the affinity of the compounds due to the increased contact surface size [31-33]. Nanotechnology is also widely applied in the process of drug encapsulation which aims to protect the active compounds of the drug and improve the mucoadhesive properties of the drug so that its bioavailability can be increased [34,35]. Nanotechnology applications in drug encapsulation can be carried out with biopolymer compounds such as chitosan. Chitosan nanoparticles are widely used because they have the advantage of being able to open epithelial tight junctions in the intestine to increase absorption in oral drugs, besides having the ideal properties of

bioavailability, biocompatibility, non-toxicity and mucoadhesive [36,37]. The aim of this study was to assess the effect of chitosan-coated pegagan nanoparticles on pro-inflammatory cytokine profiles in a chronic diabetic animal model. The cytokine profiles assessed in this study were TNF-α from helper T cells (CD4+TNF-α+) and from cytotoxic T cells (CD8+TNF-α+); IFN-γ from helper T cells (CD4+IFN-γ+) and from cytotoxic T cells IL-6+.

Methods

Study design and setting
An experimental study was conducted in male mice (*Mus musculus*) Balb/C strain. After the mice were acclimatized for 14 days, they were divided into six groups treated with different doses of nanoparticles coated with chitosan (NPC) for 28 days. On day 29, they were scarified, and the spleen was collected. The number of cytokines (CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+, CD8+IFN-γ+, IL-6+) were measured by flow cytometry.

Extract preparation
A total of 200 g of pegagan simplicia (purchased from UPT Materia Medika Batu, Malang, East Java, Indonesia) was soaked in 70% ethanol with a ratio of 1:5 (1000 mL), then homogenized with a shaker for 24 hours, the results were filtered under vacuum using a buncher funnel. The filtrate was macerated again with 70% ethanol and homogenized with a shaker at 130 rpm for 24 hours, then concentrated using a rotary evaporator at 50°C [25].

Synthesis of chitosan-coated nanoparticles
Chitosan-coated pegagan nanoparticles were prepared according to a previous study [25], starting with dissolving 3 mL of 0.5% acetic acid glacial (Merck) in 600 mL of distilled water. Then 3 g of chitosan (Merck) and 120 mL of 0.5% sodium tripolyphosphate (Merck) solution were added. The resulting mixture was homogenized at a speed of 1000 rpm. After a homogeneous process for 10 min, 0.6 g of pegagan extract was added to the solution and homogenized again. The sample was then added with 6 mL of tween 80 (Merck) and homogenized for 90 min. The addition of tween 80 served as a stabilizer [38]. The homogeneous solution was sonicated with an amplitude of 80%, a frequency of 20 kHz, and a time span of 90 min. The resulting sonication solution was centrifuged. The resulting pellets were then put into a deep freezer. The frozen pellets were incubated for 24 h at 50°C. The dry pellets were crushed then stored before used.

Animal care and treatment
A total of 42 male mice (*Mus musculus*) Balb/C strain aged 2–3 months and weighing 25–30 g were used. The mice were acclimatized for 14 days and divided into six groups. The groups were group K+ (diabetic mice treated with distilled water), P1 (diabetic mice treated with NPC 20 mg/kgBW), P2 (diabetic mice treated with NPC 30 mg/kg), P3 (diabetic mice treated with NPC 40 mg/kg), M (diabetic mice treated with metformin 130 mg/kg) and K- (healthy mice treated with distilled water).

Mice model of chronic diabetes
Mice (groups of K+, P1, P2, P3 and M) were induced by multiple low doses of STZ intraperitoneal to make chronic diabetic models, while the K- group was only induced with distilled water [39]. For the first three days, they were injected with STZ 40 mg/kgBW daily and on day five, they were injected with 60 mg/kgBW. After being injected with STZ for five days, the mice were left for nine days without any treatment. On day 14, the blood sugar levels of mice that had been fasted for 16 hours were checked by taking blood through the tail of the mice. If the blood sugar level was still normal, below 126 mg/dL during fasting [40], an additional dose of STZ 40 mg/kgBW was provided. After ensuring that the fasting blood sugar level of the mice was above 126 mg/dL, the mice were left until day 28 to get mice with diabetic complications.

Treatment procedure
Chronic diabetic mice were then given a therapeutic solution of pegagan nanoparticles for 28 days. The preparation of the therapeutic solution was carried out by dissolving pegagan

nanoparticles coated with dry chitosan with citrate buffer. The solution was given orally to mice once per day at the prescribed dose of 1 cc using gastric sonde.

Mice dissection and spleen extraction

On day 29, all mice groups were sacrificed by neck dislocation and then dissected. The mice were placed on a surgical board for abdominal dissection, and the spleen was collected. The spleen was washed 2–3 times with sterile PBS (Thermo Scientific), then cut into 1×1 cm and mashed. The smooth spleen was added with 5 mL of sterile PBS and filtered with a wire filter. The filter results were stored for the flow cytometry test.

Measurement of cytokine numbers by flow cytometry

The relative number of CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+, CD8 IFN-γ+ and IL-6+ were measured using flow cytometry. The filtered mixture of spleen and sterile PBS was centrifuged for five min at 2500 rpm. The supernatant was discarded, then the pellet was added with 1 mL of sterile PBS and resuspended. The resulting suspension (50 µL) was transferred to a 1.5 mL microtube. The pellet, which had been transferred, was then added with specific antibodies (PE anti-mouse TNF-α, PE anti-mouse IFN-γ, PE-conjugated anti-mouse IL-6, anti-CD4-FITC, anti-CD8-PE; all from BioLegend, San Diego, United States), and homogenized using a vortex. Following incubation in a dark room for 20 minutes, the suspension was centrifuged for five minutes at 2500 rpm. The samples were analyzed using flow cytometry with flow cytometry FACS Calibur (Thermo Fischer, Massachusetts, United States).

Statistical analysis

Data of the relative number of the proinflammatory cytokine of TNF-α from helper T cells (CD4+TNF-α+), TNF-α from cytotoxic T cells (CD8+TNF-α+), IFN-γ from helper T cells (CD4+IFN-γ+), IFN-γ from cytotoxic T cells (CD8+IFN-γ+) and IL-6+ were analyzed with CellQuest software (Besancon, France). In addition, the data were analyzed to determine the normality and homogeneity of the data, followed by an analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). All tests were performed using SPSS 25.0 (SPSS, New York, USA).

Results

CD4+TNF-α+ profile

The mean percentage of the relative number of CD4+TNF-α+ cells in each treatment group as followed: K+ (0.31±0.003%), P1 (0.14±0.004%), P2 (0.07±0.004%), P3 (0.12±0.006%), M (0.06±0.003%), and K- (0.12±0.006%), with the highest relative number in the K+ group (**Figure 1**). The DMRT analyzed indicated that the percentage of the relative number of CD4+TNF-α+ of P1, P2, P3 and M groups were significantly different from the K+ group (untreated chronic diabetic mice) and were not significantly different from the K- group (healthy mice) (**Figure 2**). This indicated that the administration of pegagan nanoparticles in doses of 20, 30 and 40 mg/kg reduced the percentage of the relative number of CD4+TNF-α+ cells as in healthy mice and the metformin group as a generic antidiabetic drug.

CD8+TNF-α+ profile

The percentage of the relative number of CD8+TNF-α+ were K+ (0.38%±0.005), P1 (0.22%), P2 (0.05%), P3 (0.20%), M (0.13%), and K-(0.20%) with the highest percentage of the relative number of CD8+TNF-α+ founded in the K+ group (**Figure 3**). The further test found that the K+ group was significantly different from the other groups, thus indicating an increase in CD8+TNF-α+ during chronic diabetes (**Figure 4**).
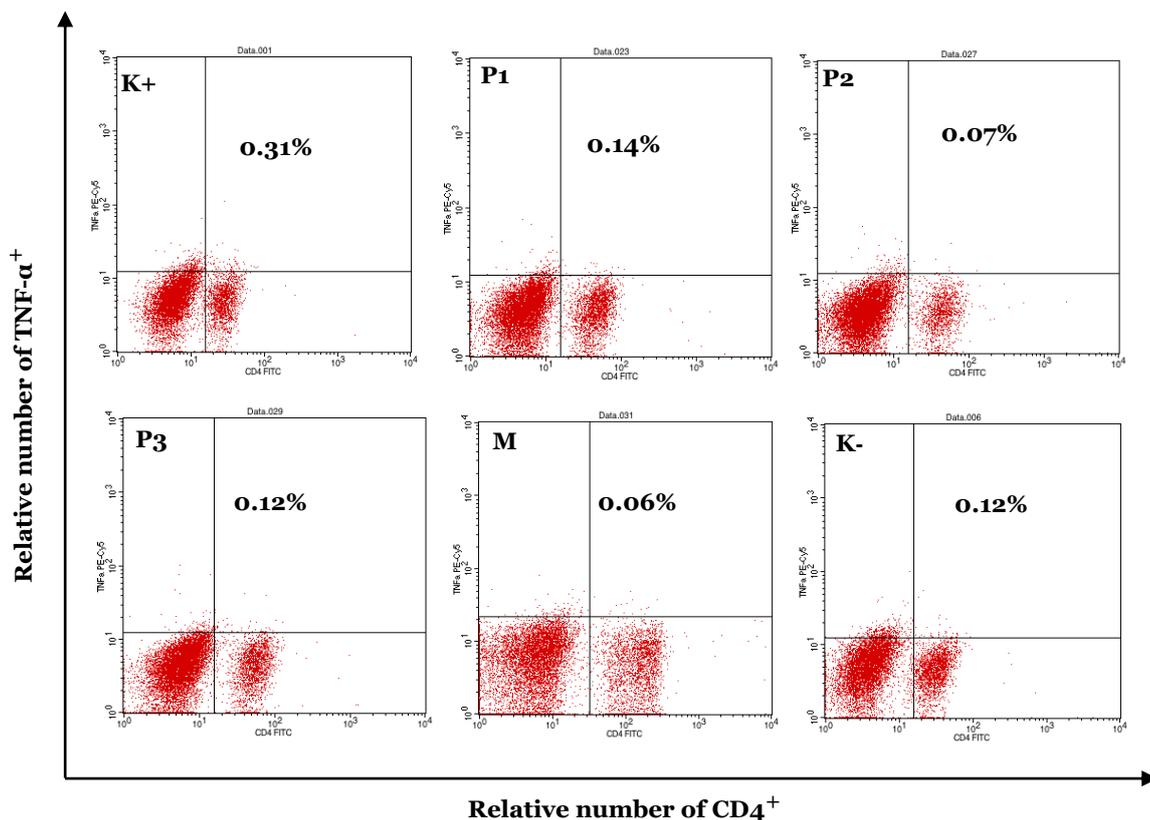
Figure 1. Flow cytometry analysis comparing the relative number of CD4+TNF-α+ cells between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-).
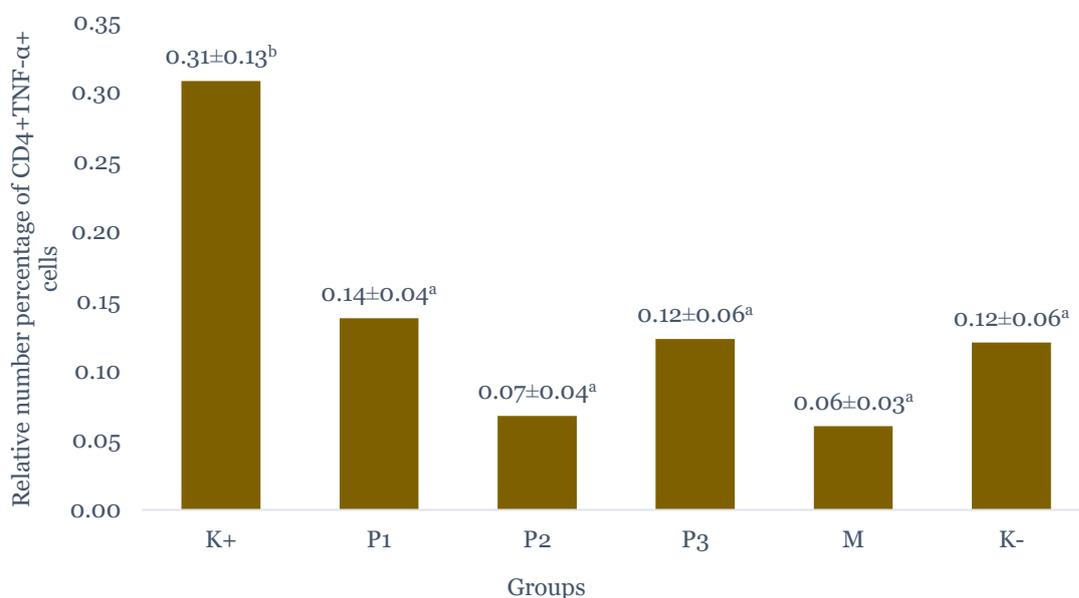
Figure 2. Comparisons of relative number percentage of CD4+TNF-α+ cells using Duncan's multiple range test (DMRT) between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-). Different letters indicate statistically significant differences ($p<0.05$).
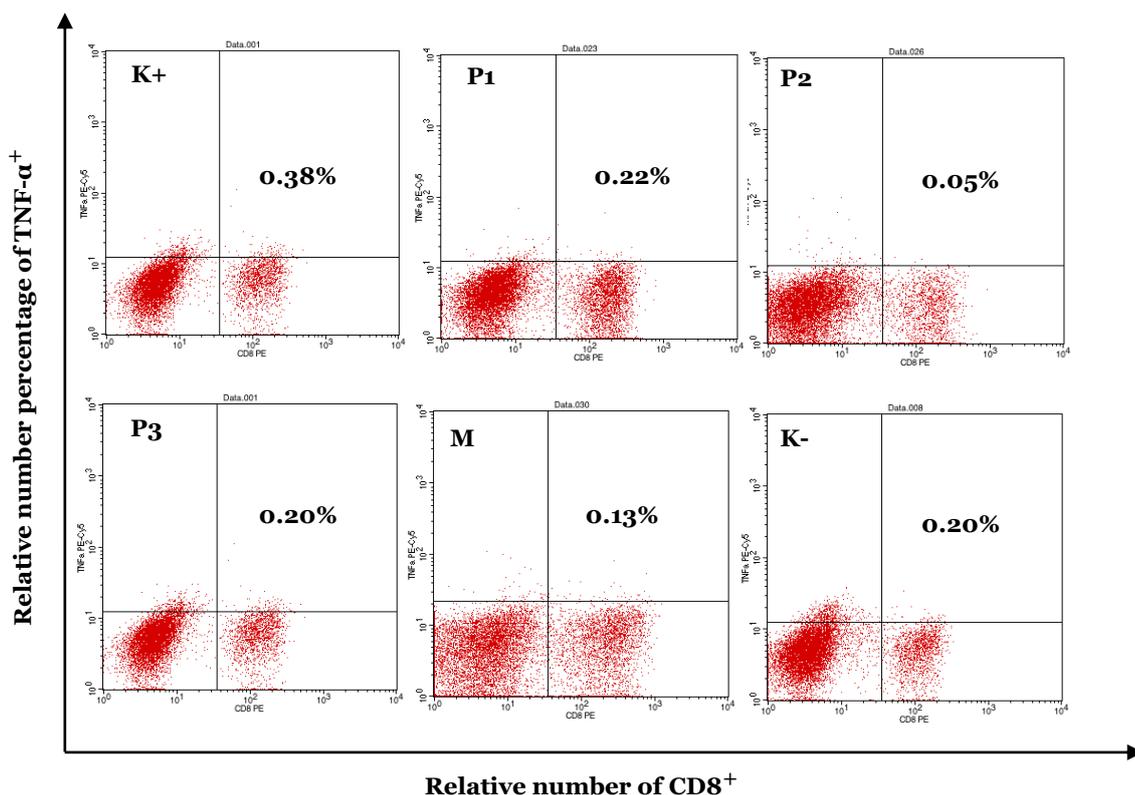
Figure 3. Flow cytometry analysis comparing the relative number of CD8+TNF-α+ cells between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-).
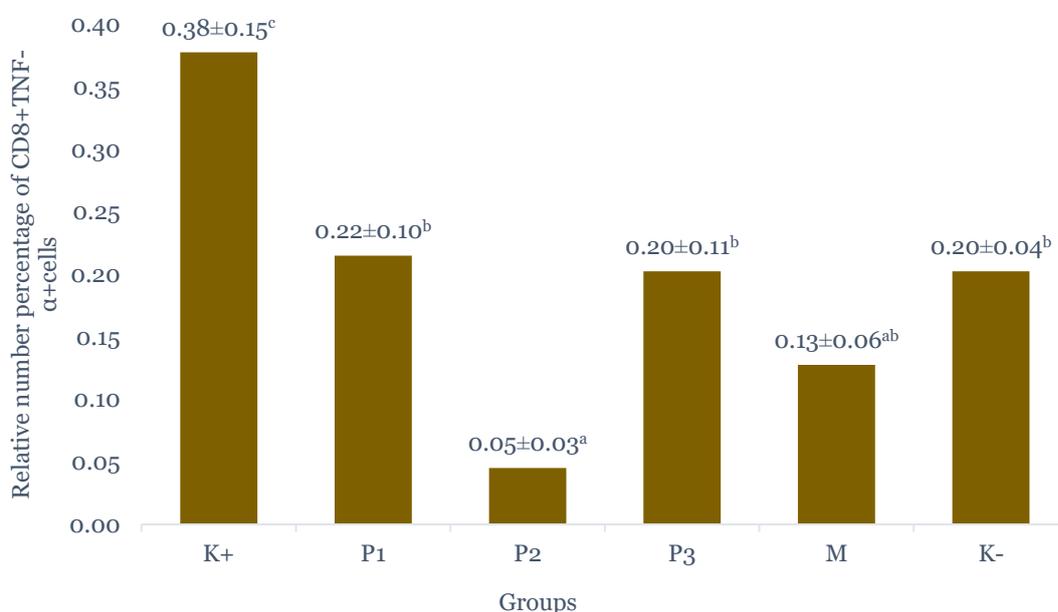
Figure 4. Comparations of relative number percentage of CD8+TNF-α+cells using Duncan's multiple range test (DMRT) between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-). Different letters indicate statistically significant differences ($p<0.05$).

CD4+IFN-γ+ profile

The average percentage of the relative number of CD4+IFN-γ+ in each treatment group is as follows: K+ (2.86%), P1 (0.61%), P2 (0.07%), P3 (0.73%), M (0.22%), and K- (0.39%) with the highest percentage of the relative number of CD4+IFN-γ+ in the K+ group (**Figure 5**). The further test results revealed that the K+ group was significantly different from the other groups, thus indicating an increase in CD4+IFN-γ+ during chronic diabetes (**Figure 6**). It also exhibited that the treatment of pegagan nanoparticles with doses 20, 30, 40 mg/kg and metformin affected on reducing the percentage of the relative number of CD4+IFN-γ+ when compared to mice in a chronic diabetic state.
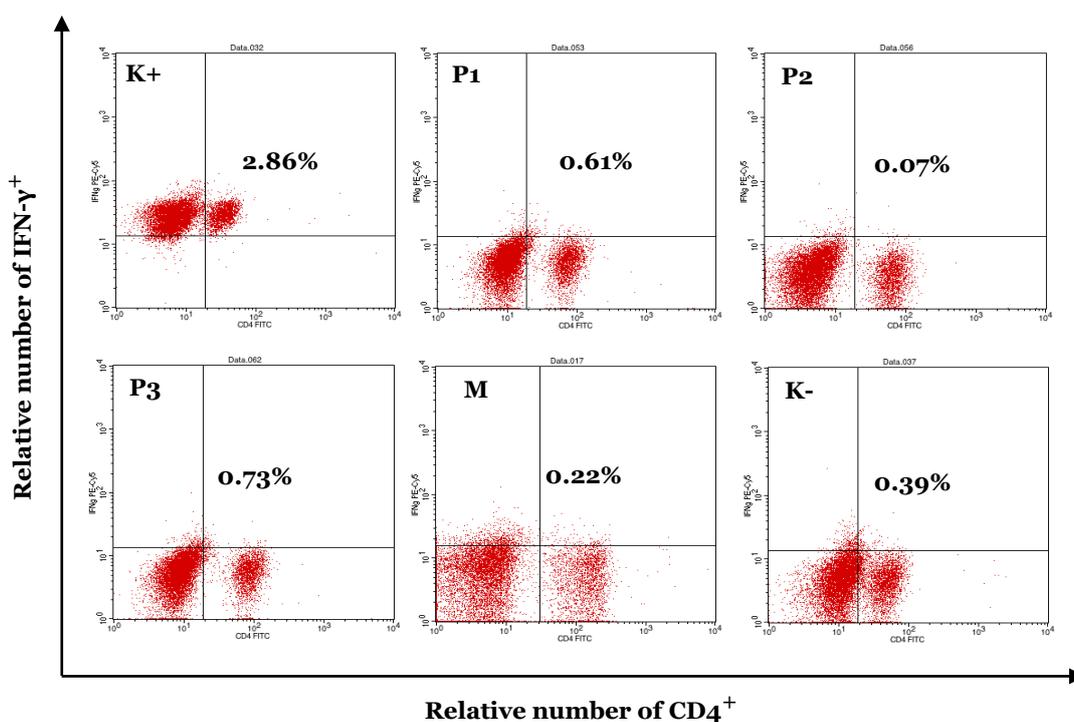
Figure 5. Flow cytometry analysis comparing the results of a relative number of CD4+IFN-γ+ cells between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-).
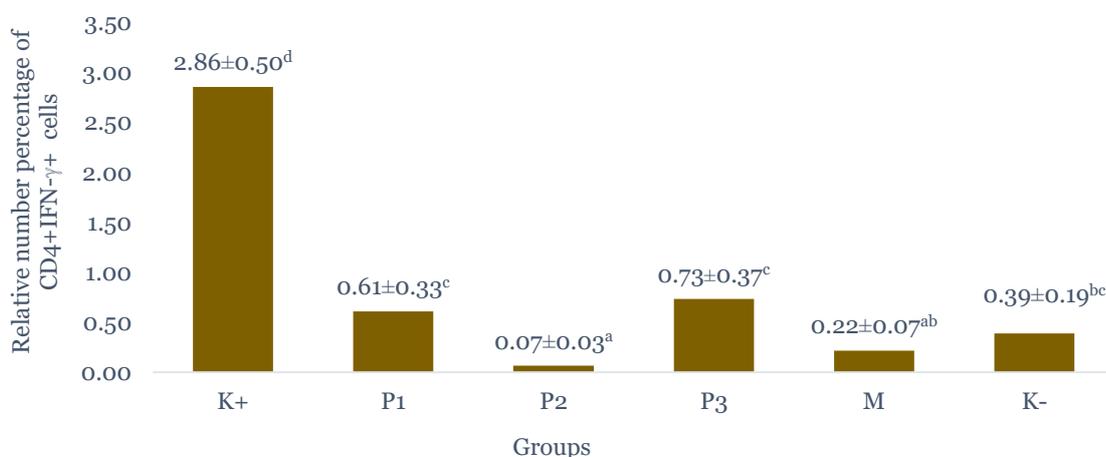
Figure 6. Comparations of the average percentage of relative number percentage of CD4+IFN-γ+ cells using Duncan's multiple range test (DMRT) between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-). Different letters indicate statistically significant differences ($p<0.05$).

CD8+IFN-γ+ profile

The average percentage of the relative number of CD8+IFN-γ+ in each treatment group (**Figure 7**) was as follows: K+ (2.55%±0.092), P1 (0.36%±0.026), P2 (0.07%±0.004), P3 (0.52%±0.025), M (0.72%±0.038), and K- (0.73%±0.028) with the highest average percentage of relative CD8+IFN-γ+ in the K+ group. The further test revealed that groups of P1, P2, P3 and M were significantly different from group K+ (**Figure 8**). Groups of Pegagan nanoparticles dose of 20, 30, 40 mg/kgBW and metformin can reduce the average percentage of the relative number of CD8+IFN-γ+.
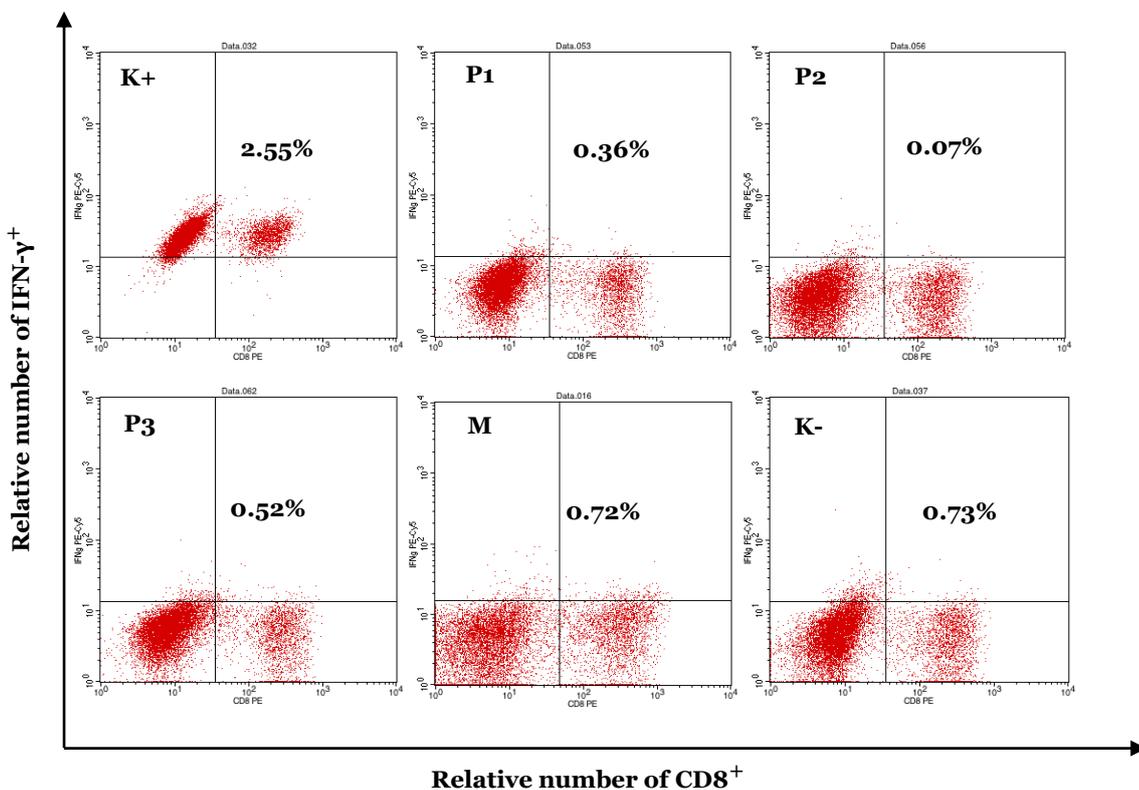
Figure 7. Flow cytometry analysis comparing the relative number of CD8+IFN-γ+cells between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-).
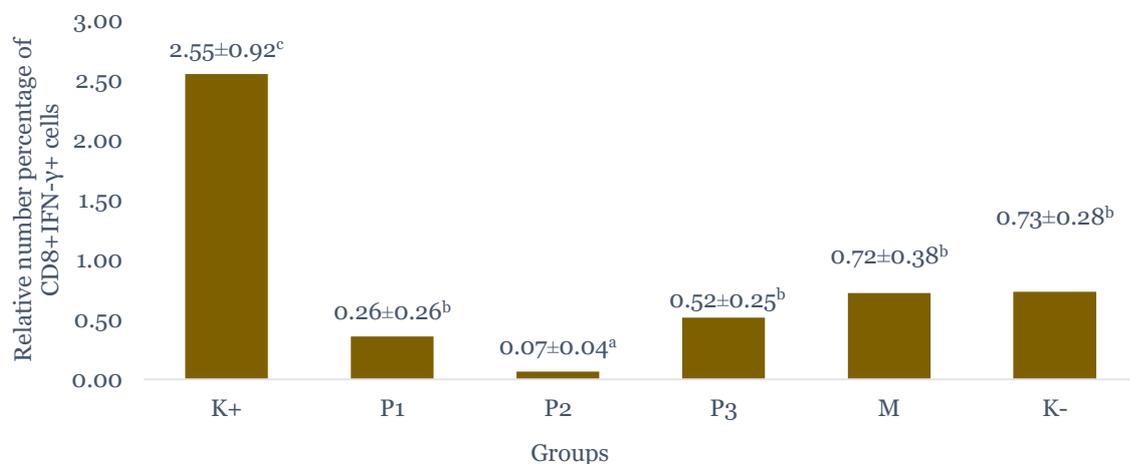
Figure 8. Comparations of relative number percentage of CD8+IFN-γ+ cells using Duncan's multiple range test (DMRT) between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-). Different letters indicate statistically significant differences ($p<0.05$).

IL-6 profile

The percentage of the relative amount of IL-6 in the spleen was carried out to determine the IL-6 profile (**Figure 9**). The following results were obtained: K+ (2.69%±0.019), P1 (1.80%± 0.057), P2 (1.45%±0.013), P3 (1.63%±0.044), M (2.20%±0.011), and K- (1.48%±0.061) with the highest percentage amount of IL-6 in the K+ group. The DMRT notation revealed that the percentage of the relative amount of IL-6 in the Pegagan nanoparticle groups has no significant difference to K- group (normal mice) and M group but has a significant difference to K+ group (**Figure 10**). This revealed that the treatment of pegagan nanoparticles up to dose of 40 mg/kgBW affected on reducing the average percentage of the relative amount of IL-6 better than using Metformin.
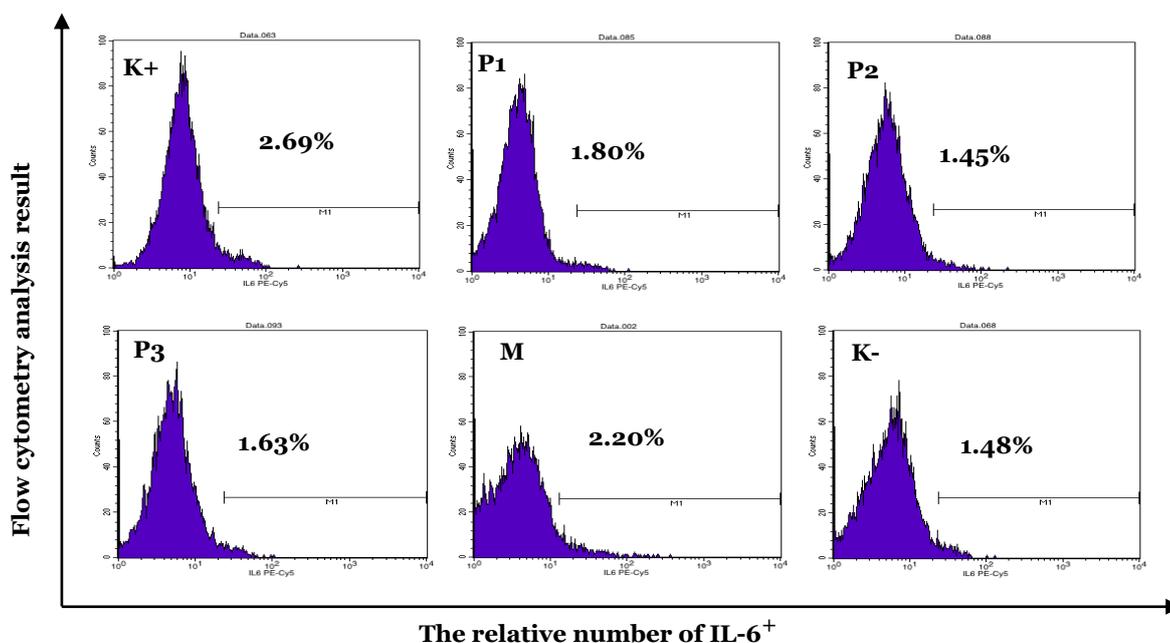
Figure 9. Flow cytometry analysis comparing the relative number of IL-6 cells between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K-).
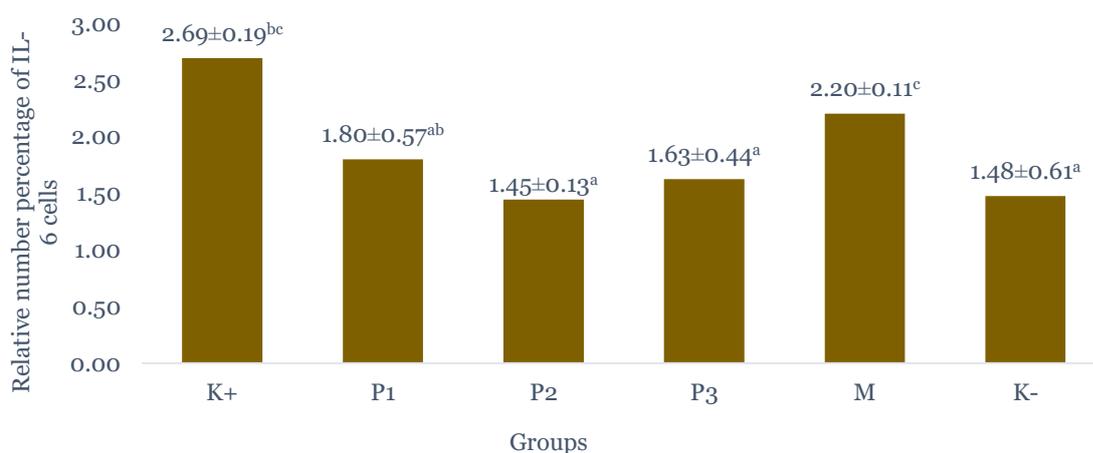
Figure 10. Comparisons of relative number percentage of IL-6 cells using Duncan's multiple range test (DMRT) between diabetic mice treated with distilled water (group K+), nanoparticle coated with chitosan (NPC) 20 mg/kgBW (P1), NPC 30 mg/kg (P2), NPC 40 mg/kg (P3), and metformin 130 mg/kg (M); and healthy mice treated distilled water (K). Different letters indicate statistically significant differences ($p<0.05$). Different letters indicate statistically significant differences ($p<0.05$).

Discussion

This study used the nanotechnology method to convert pegagan extract particles into nano-sized particles, with the aim of increasing the potency of pegagan. A previous study assessing the chitosan-coated pegagan nanoparticles with ionic gelation method using scanning electron microscopy (SEM) showed that particles have better characteristics, including having spherical particle shape, smaller and uniform particle size and particle size analyzer (PSA) tests showed that average particle size at sonification 90 minute was 286.2 nm [41]. These sizes meet the size range of polymer nanoparticles, which is between 10–1000 nm [42]. The reduced particle size of nanoparticles can optimize the solubility of active substances, improve their bioavailability, increase the stability of active substances, reduce gastrointestinal irritation due to active substances and increase the absorption of poor macromolecular compounds by the body [33].

The use of the nanotechnology method with chitosan coating is expected to protect the compounds contained in pegagan during oral administration, because chitosan is a biocompatible material that can be applied for drug delivery and various immunological activities [43]. A previous study revealed that chitosan nanomicelle used as a delivery vector modified with targeting ligands on adipocytes, is known to reduce the concentration of pro-inflammatory adipocytokines such as TNFα, MCP-1, IL-6, IL-1β, and improve insulin sensitivity in obese diabetic mice induced by a high-fat diet [44].

Pro-inflammatory cytokines were used as parameters in this study to show the inflammatory profile in chronic diabetic conditions. Inflammation plays a role in aggravating chronic diabetic conditions by causing microvascular and macrovascular damage that leads to diabetic complications [5]. CD4+TNF-α+ profile was observed in this study because the cytokine TNF-α is mostly produced by T lymphocyte cells and has the potential to be pro-inflammatory [45]. The pro-inflammatory effects of the cytokine TNF-α occur when it binds to TNF-R1, resulting in the activation of nuclear transcription factor kappa B to regulate the inflammatory response [10].

The highest average percentage of CD4+TNF-α+ was found in the K+ group, which was the treatment of mice induced by STZ. This condition can occur due to the inflammatory state in chronic diabetes. Starting with a state of high hyperglycemia that induces the production of advanced glycation end products (AGEs) through several metabolic pathways, such as the polyol pathway and the Maillard reaction [46]. AGEs can induce dendritic cell maturation and increased expression of antigen-presenting molecules, including MHC class II. It also increases the secretion of pro-inflammatory cytokines such as IL-12, which is influential in the activation and differentiation of naive CD4+T cells into Th1-type cells that can secrete pro-inflammatory cytokines such as TNF-α [47,48].

The DMRT notation of the percentage of the relative number of CD4+TNF-α+ revealed that groups treated with pegagan NPC and metformin were significantly different from the chronic diabetic mice but not significantly different from healthy mice treated with distilled water group. This exhibited that the administration of pegagan nanoparticles in doses of 20 mg/kgBW, 30 mg/kgBW and 40 mg/kgBW can reduce the average percentage of the relative number of CD4+TNF-α+ as in healthy mice and metformin-induced as generic antidiabetic, with no difference between the doses used. This anti-inflammatory action is related to the reduction of the pro-inflammatory cytokine TNF-α+, and the expression of CD4+T lymphocyte cells is associated with the compounds contained in chitosan-coated pegagan nanoparticles.

Decreasing the percentage of the relative number of CD4+TNF-α+ when treated with chitosan-coated pegagan nanoparticles may be mediated by the content of the main triterpene compounds, such as the asiatic acid compound. The mechanism of asiatic acid reduces the levels of inflammatory cytokine TNF-α significantly through the anti-inflammatory mechanism of decreasing the expression of transcription factor NF-κB [49]. Asiatic acid can also downregulate the mRNA expression levels of Th1-related cytokines such as TNF-α and IL-1β and the protein level of inflammatory cytokine IL-6, as well as the NF-κB and MAPK signaling pathways in mice [16].

The CD8+TNF-α+ profiles were observed because the adaptive immune system, especially T lymphocytes, also plays an important role in the pathogenesis of diabetes [50]. It is known that inhibition of Tc1 cells can ameliorate STZ-induced diabetes mellitus; this is because Tc1 cells, a subset of CD8+T cells, secrete IFN-γ and TNF-α, which contribute to cytotoxic activity in diabetes

[51]. The CD8+TNF-α+ profile in the K+ group, according to the DMRT follow-up test, was significantly different from the K- group, which is healthy mice, showing an increase in CD8+ TNF-α+ during chronic diabetes. This is due to the accumulation of AGEs in diabetics inducing the maturation of dendritic cells that can act as antigen-presenting cells in the activation of Tc1 cells. [48] Antigen-presenting cells, including macrophages and dendritic cells, produce the cytokine IL-12 for the activation of Tc1 cells. Tc1 cells are a CD8 subset that produces IFN-γ and TNF-α cytokines when compared to other cell subsets [9]. Treatment of pegagan nanoparticles at doses of 20, 30, 40 mg/kgBW and metformin can reduce the percentage of the relative number of CD8+TNF-α+ as in healthy mice. The anti-inflammatory action related to the decrease in pro-inflammatory cytokine TNF-α+ and CD8+T lymphocyte cell expression when treated with pegagan is associated with the content of asiatic acid in *C. asiatica*. Asiatic acid can inhibit the mRNA expression of pro-inflammatory cytokines, including TNF-α and IL-1β, through suppressing NF-κB activation [52]. Quercetin compound also has anti-inflammatory actions by inhibiting the production of IL-12 and inhibition of JAK2 and STAT4, resulting in decreased IL-12-induced T cell proliferation and differentiation [53].

The diabetic mice treated with NPC 30 mg/kgBW) group revealed that the relative number of CD8+TNF-α+ was significantly different results from the K- group (healthy mice), with the average P2 value being lower than the K-, but was not significantly different with metformin induced. This shows that the administration of chitosan-coated pegagan nanoparticles at a dose of 30 mg/kgBW has an effect on reducing the percentage of the relative number of CD8+TNF-α+, which is lower than the healthy mice group. Whether the decrease has an adverse effect on the regulation of the immune response or the decrease is still within the normal range for CD8+TNF-α+ still needs further study.

Chitosan-coated pegagan nanoparticles with the smallest dose (20 mg/kgBW) were able to reduce the percentage of the relative number of CD8+TNF-α+ as in healthy mice and metformin administration, so that the dose can be used as a substitute for standard metformin drugs. The use of metformin has the contradiction of causing severe liver function disorders and can increase the risk of lactic acidosis when accumulated in the body [54].

The observation of CD4+IFN-γ+ and C8+IFN-γ+ profiles in this study was carried out to see how the state of T helper cells and pro-inflammatory cytokine IFN-γ+ are expressed when in a state of chronic diabetes and when given chitosan-coated pegagan nanoparticles. CD4+IFN-γ+ and CD8+IFN-γ+ were chosen because the adaptive immune system, especially T lymphocytes, play an important role in the pathogenesis of diabetes [50].

When CD4+T cells are activated in the presence of IL-12, they differentiate into Th1, secreting the cytokine interferon-γ (IFNγ), and several subsets of CD8 T cells have been identified, including Tc1, which produces the cytokines IFN-γ and TNF-α [9]. Th1, Th2 and CD8+ cells are the source of various chemokines, cytokines and adipokines that modulate inflammation in obesity and diabetes [55].

An increase in CD4+IFN-γ+ during chronic diabetes can occur because, in chronic diabetes, there is an accumulation of AGE. When AGEs bind to RAGE (AGE receptor), it can activate dendritic cells that act as antigen-presenting cells in the activation and differentiation of CD4+ T lymphocytes [56]. The activity of the signal transducer and activator of transcription (STAT 1) signaling serves as a key regulator of Th1 differentiation. Th1 cells are a subset of CD4 cells that express the IFN-γ cytokine [57].

The CD4+IFN-γ+ profiles of P1, P3 and M groups were not significantly different from group healthy mice. This shows that pegagan nanoparticles at a dose of 20 mg/kgBW can reduce the average percentage of the relative number of CD4+IFN-γ+ as in healthy mice. This decrease is mediated by asiaticoside compounds that are known to suppress inflammatory response by suppressing phosphorylation of NF-κB and degradation of its inhibitor IκBα [58]. Flavonoid compounds such as kaempferol and quercetin are also known to inhibit STAT 1, which plays a role in CD4+T cell differentiation and can inhibit NF-κB activation [59].

The increase in CD8+IFN-γ+ during chronic diabetes, as shown in the research results, can occur because, in chronic diabetes, there is hyperglycemia or high blood glucose levels that can induce an inflammatory state characterized by an increase in IFN-γ+ cytokines produced by CD8+ T cells. This is not contradictory to previous research, which revealed that STZ-induced

complications in a diabetic mice model showed infiltration of CD4+ and CD8+T cells in the renal interstitial of diabetic mice. IFN-γ and TNF-α levels were also increased significantly in the kidneys of diabetic mice when compared to control mice [16].

The percentage of the relative number of CD8+IFN-γ+ showed that the P1, P3 and M groups were not significantly different from the K- group (healthy mice). The anti-inflammatory action associated with the decrease in pro-inflammatory cytokine IFN-γ+ and CD8+T lymphocyte cell expression is associated with the content of compounds contained in pegagan. Asiaticoside was found to prevent diabetes-related cognitive deficits by suppressing the NF-κB pathway as a transcription factor for inflammatory mediators [60]. The flavonoid compound Kaempferol, which can also be found in pegagan, can suppress mononuclear cell infiltration, IL-12, iNOS, and NF-κB activation, IκBα phosphorylation in renal tissue nephrotoxicity due to cisplatin induction. It is known that IL-12, NF-κB activation and IκBα phosphorylation play a role in the differentiation of CD8+ T cells into Tc1 cells that produce pro-inflammatory cytokine IFN-γ+ [61]. Emphasis on this mechanism is thought to reduce the average percentage of the relative number of CD8+ IFN-γ+.

The IL-6 cytokine profile was observed because diabetes can be expressed as a chronic form of autoinflammatory disease. Potential inflammatory responses that play an important role in inflammatory mechanisms in the pathogenesis of diabetes are mediated by many pro-inflammatory cytokines such as IL-6 [62]. IL-6 is produced by monocytes, fibroblasts, and endothelial cells [10]. The pro-inflammatory effects of IL-6 assist monocyte recruitment by increasing chemokines that attract monocytes [63].

The highest percentage of the relative amount of IL-6 among all treatments was found in group K+, which is the treatment of mice induced by STZ to make chronic diabetes without being treated with chitosan-coated pegagan nanoparticles. This is in line with a previous study that revealed an increase in TNFα, IL-6 and IFN-β levels in neutrophils of people with diabetic complications when compared to normal people [5].

The treatment of pegagan nanoparticles with doses of 30 and 40 mg/kgBW affected reducing the percentage of the relative amount of IL-6 when compared to mice with chronic diabetes. The decrease in the pro-inflammatory cytokine IL-6 can be associated with the main triterpene asiatic acid content of *C. asiatica,* which can provide anti-inflammatory effects by inhibiting the production of inflammatory cytokines TNF-α, IL-6, and IL-1β which are mediated through blocking NF-κB activation [64].

The average IL-6 in metformin treatment at a dose of 130 mg/kgBW did not show a significant difference from the average in chronic diabetic mice. This is likely caused by the need for a longer period of administration of metformin to have a decreasing effect on IL-6. As per previous study findings, treatment with metformin 1500 mg/kgBW for six months could reduce TNF-α levels significantly but does not reduce IL-6 levels significantly, while a significant decrease in IL-6 levels was observed after metformin treatment for 12 months [65].

Conclusion

The administration of pegagan nanoparticles coated with chitosan significantly reduced the relative number of pro-inflammatory cytokines (CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+ and CD8+IFN-γ+ and IL-6) at doses ranged between 20 and 40 mg/kgBW. The smallest dose of 20 mg/ kgBW could reduce CD4+TNF-α+, CD8+TNF-α+, CD4+IFN-γ+, CD8+IFN-γ+ to the level of healthy mice and a dose of 30 mg/kg reduced IL-6+ to the level of healthy mice.

Ethics approval

Acknowledgements

Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

Muchtaromah B, Firdaus AMK, Ansori ANM, *et al.* Effect of pegagan (*Centella asiatica*) nanoparticle coated with chitosan on the cytokine profile of chronic diabetic mice. Narra J 2024; 4 (1): e697 - http://doi.org/10.52225/narra.v4i1.697.

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