Heliyon

Immunomodulatory effects of black solo garlic (Allium sativum L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract:	Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1b (IL-1b) and interferon gamma (IFN-g) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1b, IL-6 and TNF-a as well as increased IFN-g with the immunity of STZ-induced rats.
Opposed Reviewers:	

Manuscript Review

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Reviewer's Comments:

Reviewer #1: The manuscript offers a new insight into the immunomodulatory activity of black garlic in diabetic animal models. However, some points need to be improved and/or clarified as follows:

Introduction

* *Why did authors measure IL-1, IL-6, TNF-α, and IFN- as the parameters in the immunomodulation of diabetic animal model? A reference for using those cytokines and chemokines will improve the introduction section.*

Response: We have added information about the relationship of inflammatory cytokines IL-1β, IL-6, TNF-α, as well as IFN-γ in diabetes (Ref 4,5) in the immunomodulation in Introduction.

Ref [4] Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57: 136–142. doi:10.1016/j.cyto.2011.09.029

[5] Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. J Inflamm Res. 2020;13: 187–193. doi:10.2147/JIR.S245064

Methods:

* *Line 59 → the modified vehicle → what is this modified vehicle?*

Response: vehicle means equipment that are needed for making black solo garlic. We change to "fermentation modified apparatus" refer to rice cooker.

* *Line 81 → steeping black solo garlic was given per oral → in what vehicle?*

Response: We did not use any vehicle because the solution is available as "steeping" form.

* *Section 2.2 → When was glibenclamide given (same schedule as extract?)? Further, it's not clear if the treatment was performed every day. Please clearly state this.*

Response: Yes, positive control was given in the same schedule with treatment group. Now we have added the information about schedule of glibenclamide on manuscript.

* *Section 2.4 → statistical analysis: using ANOVA? From the graph, it looks like each set of data (pre- and post-) was analyzed using paired t-test? If ANOVA was used, then authors also can compare the post-treatment data to the 'normal' group? Please clarify.*

Response: We re-analyzed for statistical analysis using Two-way ANOVA with a Tukey post-hoc test. Yes, we now compare the post-treatment data with other group data, including normal group. See the Figure 2 and 3 for new statistical analysis result.

Results and interpretation

* *Was there no identification of extract's active content? This will lead to a better discussion in the manuscript.*

Response: Yes, we did GC-MS to compare the content of fresh solo garlic and black solo. garlic.

* *Line 103: confusing sentence explaining the dose. Please rephrase*

Response: We modified to: “Therefore, treatment of the black solo garlic dose of 13.5 g/kg body weight are equal to those 26 g/kg body weight as an anti-inflammatory agent.”

* *Line 106-107: please rephrase the sentence. Better to divide into 2 sentence for better clarity*

Response: We split into two sentences as suggested: “Streptozotocin induction reduced IFN-gamma level in the rats. In contrast, black solo garlic in all doses significantly increased IFN-gamma level”

Related to statistical analysis (please also see last point in Method above)

* *Line 100: The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1 level more significant than those of glibenclamide → but there's no statistical analysis (in the figure) directly comparing two groups*

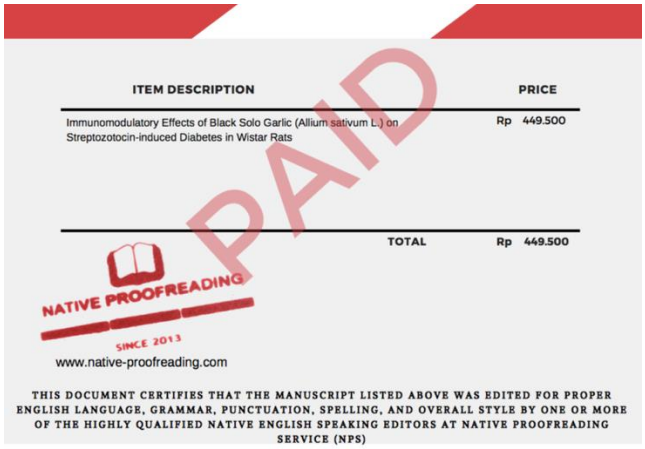
Response: We have added in the new Figure.

* *Line 108: Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the increased of IFN- levels than STZ-induced only group → but in figure, there's no direct statistical analysis between these groups.*

Response: We have added in the new Figure.

Lastly, the manuscript needs a professional English proofreader to improve the readability of the text.

Response: We have consulted to proofreading service.



Reviewer #2

I- Decision:

The article should be rejected as it needs major experimental and analytical work and revision of scientific information as per the stated comments.

II- General Comments:

Although, I do admire the good intentions of the authors for investigating the immunomodulatory actions of black solo garlic (BSG) in the model of STZ-Induced type-1 diabetes, nonetheless there is a lot that is missing to make this study credible and worth consideration for publication. I am sorry to say that the scientific argument presented, experimental and analytical work done, data presented and conclusion(s) reached do not mount to what is found even in a weak scientific poster presented by undergraduate students.

Response: Thank you and we have modified many parts as comments suggested. Although this study is quite simple, the study about black solo garlic (BSG) is important to add the effectiveness of BSG in inflammation field (in general) and diabetes (in specific).

III- Abstract:

The information presented in the abstract reflects the standard of the work done.

IV- Introduction: Is lacking major details and information.

In general, the scientific information presented in the introduction is superficial, inadequate and in certain places hardly relevant to and does not ideally set the scene for a logical and credible justification of why this study should be carried out (support the objective(s) and aim(s) of the study):

1. *The description of the condition of diabetes does not present accurate details of the subtypes.*

Response: Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin

deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required.

Ref. [3] Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. Int J Vasc Med. 2012;2012: 918267. doi:10.1155/2012/918267

2. *The casual connection made between the expected effects of treatment with BSG and the variables of inflammation leaves a lot to be desired. Also check the correct scientific name of solo garlic.*

Response:
Solo garlic known as single clove garlic, monobulb garlic, single bulb garlic, or pearl garlic is a type of *Allium sativum*. Scientific name of solo garlic: *Allium sativum* 'Solo garlic'. Diabetes is associated with high levels of cytokines TNF-alpha, IL-6, IL-1beta, and decreased interferon-gamma [4,5]. Single black garlic contains flavonoids as antioxidants and anti-inflammatory.

Ref [4] Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57: 136–142. doi:10.1016/j.cyto.2011.09.029

5. Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. J Inflamm Res. 2020;13: 187–193. doi:10.2147/JIR.S245064

3. *The reason(s) for choosing the selected (tested) inflammatory and anti-inflammatory variables (among many which have an effect on inflammatory responses) is not adequately stated. This point should be clarified and supported. Also the authors did not state the pleiotropic effects of the immune variables tested and how their modulations serves the objectives of the study.*

Response: Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ (Mirza et al. 2012; Kartika et al. 2020). TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production (Ortiz et al. 1995). IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy (Chang and Chuang 2010). IL-1 is a master cytokine of inflammation (Dinarello and van der Meer 2013).

Ref [4] Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57: 136–142. doi:10.1016/j.cyto.2011.09.029

5. Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. J Inflamm Res. 2020;13: 187–193. doi:10.2147/JIR.S245064
6. Ortiz A, Bustos C, Alonso J, Alcázar R, López-Armada MJ, Plaza JJ, et al. Involvement of tumor necrosis factor-alpha in the pathogenesis of experimental and human glomerulonephritis. Adv Nephrol Necker Hosp. 1995;24: 53–77.
7. Chang Y-C, Chuang L-M. The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication. Am J Transl Res. 2010;2: 316–331.

4. *A comparison between the content of aged regular garlic (multicloved) and BSG (single cloved) should be stated to highlight the significance of using extract of BSC in this study. Aged regular garlic also contains high amounts of remedial agents compared to fresh regular garlic.*

   Response: Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, SAC, and alkaloids content higher than those of fresh garlic [9]. black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14].

V- Objective(s) – Aim(s): Is lacking in details and information

A separate objective(s) – Aim(s) section is missing. If stating such sections is in tune with the journal guidelines for publication of articles, such section should be included.

Response: We added in the introduction.

VI- Methodology: Is lacking in details and information and more importantly significant steps and procedures.

1. *The methodology section needs to be divided into subsection for better presentation of the listed procedural steps.*

Response: Thank you. The new list has been made in the manuscript.

2. *The procedure describing the preparation – fermentation of fresh solo garlic to get BSG is vague. Was any liquid (such salted-water or vinegar, ethanol, etc.) or any other chemical added to fresh solo garlic to induced fermentation (or was it only dry fermentation)? Was a single batch of solo garlic fermented or more than one batch. If more than one batch was fermented, how the batches contents were standardized. Did the authors carry out GS-MS analysis to identify the different contents of this 'new' preparation to show its presumed and suggested active components content?*

Response: It's only dry fermentation. We used an 800 mL volume rice-cooker for fermentation so one batch fermentation is enough for sample's experiment. Yes, we did GC-MS to compare the content of fresh solo garlic and black solo garlic.

3. *What was the dose(s) of BSG that were administrated? What was the concentration of BSG in the seeping extract used for treatment?*

Response: We gave three doses for three different group: (i) 6.5, (ii) 13.5, and (iii) 26 g/kg body weight. BSG was mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 °C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

4. *How the efficacy of hyperglycaemia induction by STZ was tested to prove inflection of diabetes in the treated (and non-treated groups). Also, did the authors measure the concentration of glucose to ascertain the antidiabetic efficacy of BSG.*

Response: Yes, blood sugar was checked the second day after STZ induction.

5. *Did the authors measure the preliminary parameters (such as body weight, food and water intake and urine output – which are general indicators of diabetes) to show inflection of diabetes (in response to STZ) and recovery (in response to BSG)? Is the acute treatment with BSG (7-10 days) is enough to induce the effect of this preparation. Many variables were not measured and it is hard to ascertain this outcome. For positive control, the authors should have used more appropriate antidiabetic agents against type-1 (such as continuous insulin administration by mini-osmotic pumps or scheduled treatment with metformin). The use of glibenclamide for positive control is not appropriate as this antidiabetic drug is mostly used to treat type-2 diabetes. This drug stimulates the release of insulin from pancreatic beta-cells by depolarization. The suggestion that this drug is effective in the authors' model of STZ-induced (beta cell destruction) type 1 diabetes indicates that STZ was not effective. The measurement of insulin could have supported the arguments of the authors and the antidiabetic efficacy of their BSG preparation*

   Response: Yes, all parameters were recorded. Treatment of BSG for 8 days can suppress the inflammatory cytokines as shown in the Results. Those suggested methods such as continuous insulin administration by mini-osmotic pumps is difficult to be applied, and what we did already stated in the methods. Yes, we know the mechanism action of glibenclamide, and we want to see the protective effect glibenclamide in STZ-mice model. If the glibenclamide works, it does not mean the STZ-model was failed. Glibenclamide is commonly used in that model, for example in the https://journals.sagepub.com/doi/full/10.1177/1470320312460881 and https://www.sciencedirect.com/science/article/pii/S2211383512000263 and https://www.karger.com/Article/Fulltext/496104.

6. *The protocol of cytokines estimation needs further elucidation.*

   Response: We have added more detail information about this assay in the Method.

7. *As per the groups devised and treatments carried out a two-way ANOVA with a TuKey post-hoc test would have been more appropriate.*

Response: Thank you for your suggestion. We did statistical analysis with two-way ANOVA with a Tukey post-hoc test and the result shown in the Figure 2 and 3.

VII- Results: Is lacking in detail and superficial as a result of the simplicity of analysis carried out.

1. *Comparison between the groups is superficial and does not show the percentage changes in response to each experimental manoeuvre.*

   Response: Now we mentioned it in the text (Result section paragraph).

2. *The graphs included are redundant.*

   Response: A new Figure has been drawn that show significant between STZ-induced group with treatment group. (Figure 2 and 3)

VIII- Discussion: is lacking in details and information.

1. *The stated information and level of argument is superficial, disjointed and even loosely related. Listing scientific information just for the sake of it does not support and provide merited and well-founded discussion.*

   Response: We re-arrange the discussion part.

2. *The argued mechanisms of increase (due to the induction of diabetes) and decrease (due to treatment with BSG) in inflammatory and anti-inflammatory variables is flimsy.*

   Response: We re-arrange the discussion part.

IX- Conclusions:

The couple of statements listed can hardly be considered as a conclusion. They are no more than restating what was already mentioned in the result section.

Response: We delete this section and elaborate in the discussion part.

Bibliography

Chang, Y.-C. and Chuang, L.-M. 2010. The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication. *American journal of translational research* 2(3), pp. 316–331.

Dinarello, C.A. and van der Meer, J.W.M. 2013. Treating inflammation by blocking interleukin-1 in humans. *Seminars in Immunology* 25(6), pp. 469–484.

Kartika, R., Purnamasari, D., Pradipta, S., Larasati, R.A. and Wibowo, H. 2020. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. *Journal of inflammation research* 13, pp. 187–193.

Mirza, S., Hossain, M., Mathews, C., Martinez, P., Pino, P., Gay, J.L., Rentfro, A., McCormick, J.B. and Fisher-Hoch, S.P. 2012. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. *Cytokine* 57(1), pp. 136–142.

Ortiz, A., Bustos, C., Alonso, J., Alcázar, R., López-Armada, M.J., Plaza, J.J., González, E. and Egido, J. 1995. Involvement of tumor necrosis factor-alpha in the pathogenesis of experimental and human glomerulonephritis. *Advances in nephrology from the Necker Hospital* 24, pp. 53–77.

Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

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is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). F~~f~~ermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. ~~Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [5].~~Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic' ~~L.~~), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, ~~fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [4]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [5].~~

b~~B~~lack solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. ~~{...}[7]{...}The previous study showed that black ordinary garlic extract increases the immune system [8] and recover kidney cells [9].~~ However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore,

this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory genes (TNF-α, IL-6, IL-1β, IFN-γ) expression to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

~~**2.1. reparation of steeping black solo garlic**~~

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus~~vehicle~~ by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days.

After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic~~, and~~ were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 °-C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

~~2.2.~~ 2.4. Animals ~~and Treatment~~

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used.

2.5 Streptozotocin-induced diabetic rats

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

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2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0,09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5, (v) 13.5, and (vi) 26 g/kg body weight.

~~Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction~~A~~, and a~~fter the induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19th. The same schedule was also applied for glibenclamide treatment (Figure 1). S~~Also, s~~teeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

~~2.3.~~ 2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits~~s~~ were~~as~~ used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. ~~, using the Reader (Labotrone, Germany)~~Briefly, t~~,~~his ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

~~2.4.~~ 2.8. Statistical analysis

All data were presented as mean ± SEM and G~~g~~raphPad Prism 8 (California, US) was~~ere~~ used to generate the figure. Furthermore, the mean scores between the treatment groups were compared with the two~~one~~-way ANOVA with a Tukey post-hoc test~~and least significance different (LSD) test~~. p<0.05 was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level in comparison to the normal group (Figure 2). The administration of black solo garlic at dose of 13.5 g/kg and 26 g/kg reduced 50% the level of IL-6~~,~~ and reduced 37% the level of IL-1β, and TNF-α ~~IL-1β, and TNF-α~~ than STZ-induced only group. The

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treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1β level more significant than those of glibenclamide (Figure 2B). Moreover, the treatment of black solo garlic was able to reduce TNF-α levels in all doses (Figure 2C). Therefore, treatment of the black solo garlic ~~treatment~~ dose of 13.5 g/kg body weight are equal ~~with~~ to those 26 g/kg body weight ~~acts~~ as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction reduced IFN-γ level in the rats. In contrast, ~~In the treatment of STZ and~~ black solo garlic in all doses increased~~,~~ IFN-γ ~~was reduced and increased respectively~~level (Figure 3). Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN-γ levels than those of STZ-induced only group~~p~~ ~~(Figure 3)~~.

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [15], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [16]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [17]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling [18]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [19]. Flavonoids can function as antioxidants to reduce or terminate free radical chain reactions [20]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [21]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [22,23].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [24]. These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [25,26]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that help to generate IL-1β is interesting to be explored in the future study.

~~**Conclusion**~~

~~The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. Therefore, it can be used as an immunomodulator.~~

APPENDICES

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

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Figures

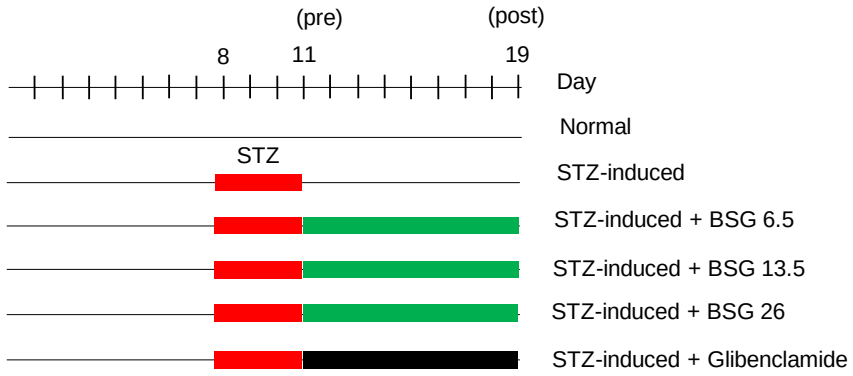

Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo

garlic.

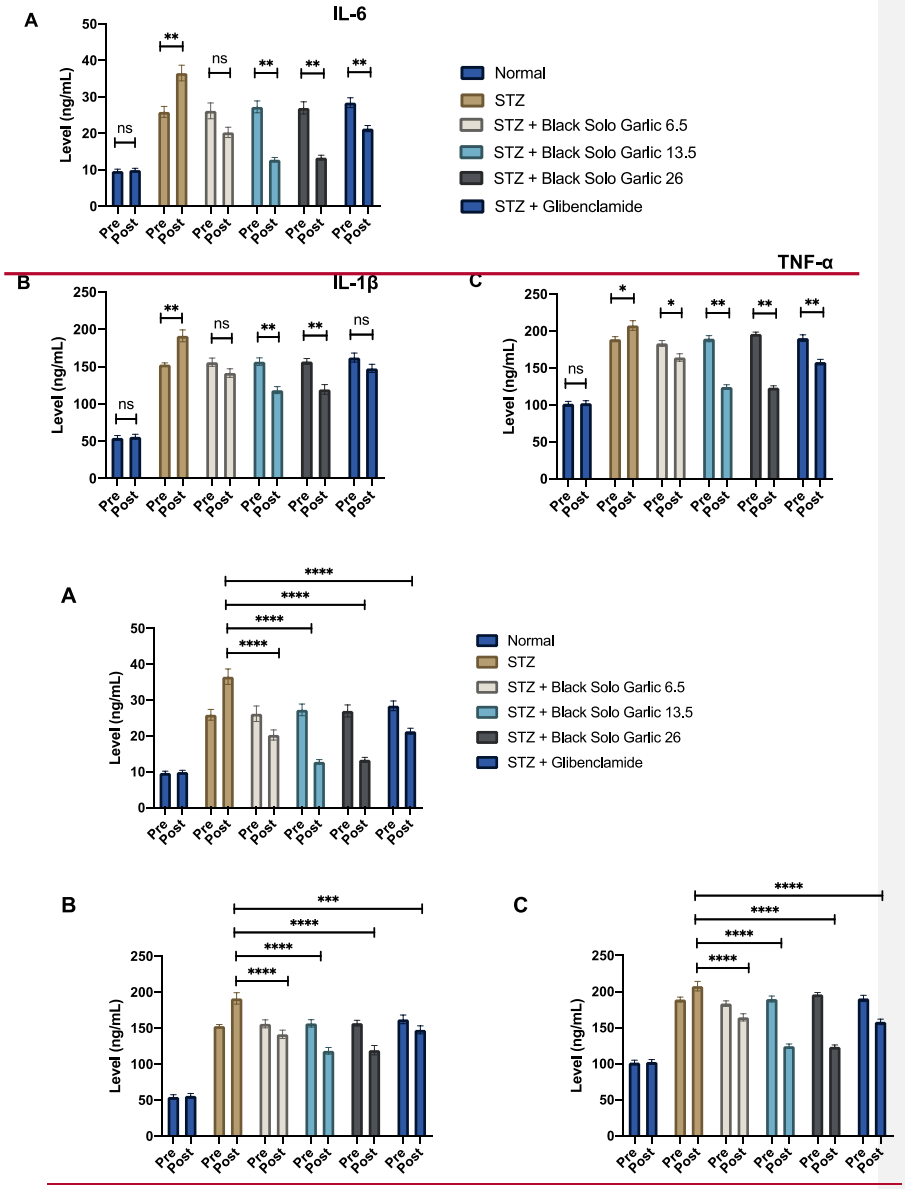

Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6 ~~level~~, (B) IL-1β ~~level~~, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were determined by ELISA. ** p <0,0001; * p<0,00055; ~~ns = not~~

~~significant.~~

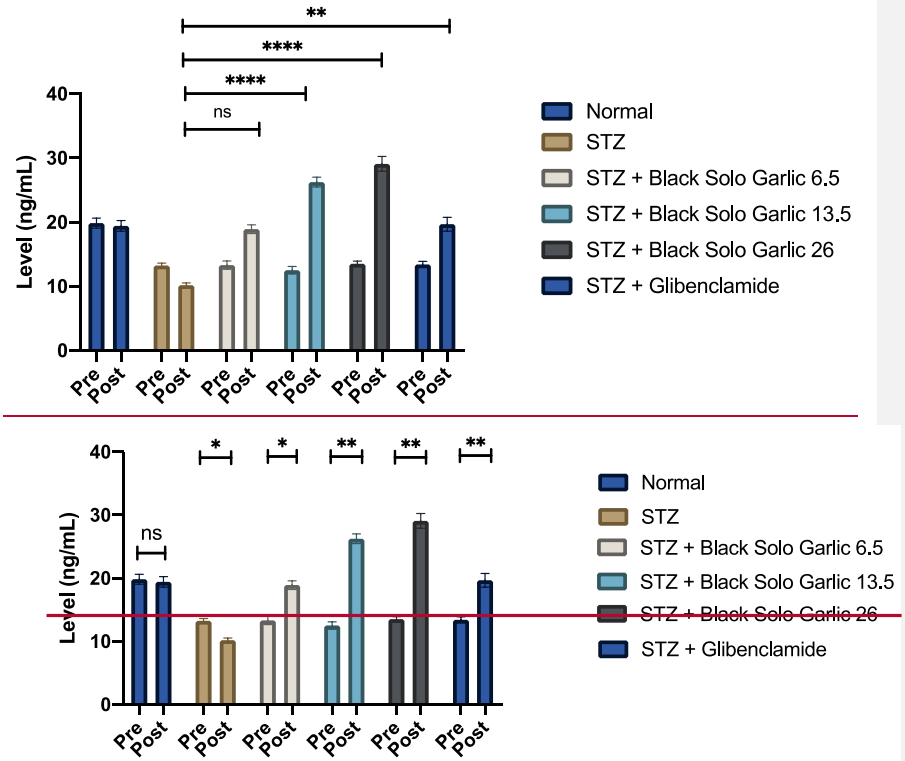

Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats was determined by ELISA. **** p<0,0001; ** p <0,01; ~~* p<0,05;~~ ns = not significant.

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). Fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic'), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory genes (TNF-α, IL-6, IL-1β, IFN-γ) expression to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days. After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 °C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

2.4. Animals

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used.

2.5 Streptozotocin-induced diabetic rats

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0,09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5, (v) 13.5, and (vi) 26 g/kg body weight. After the induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19$^{th.}$ The same schedule was also applied for glibenclamide treatment (Figure 1). Steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits were used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. Briefly, this ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

2.8. Statistical analysis

All data were presented as mean ± SEM and GraphPad Prism 8 (California, US) was used to generate the figure. Furthermore, the mean scores between the treatment groups were compared with the two-way ANOVA with a Tukey post-hoc test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level in comparison to the normal group (Figure 2). The administration of black solo garlic at dose of 13.5 g/kg and 26 g/kg reduced 50% the level of IL-6 and reduced 37% the level of IL-1β, and TNF-α than STZ-induced only group. The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1β level more significant than those of glibenclamide (Figure 2B). Moreover, the treatment of black solo garlic was able to reduce TNF-α levels in all doses (Figure 2C). Therefore, treatment of the black solo garlic dose of 13.5 g/kg body weight are equal to those 26 g/kg body weight as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction reduced IFN-γ level in the rats. In contrast, black solo garlic in all doses increased IFN-γ level (Figure 3). Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN-γ levels than those of STZ-induced only group.

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [15], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [16]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [17]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling [18]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [19]. Flavonoids can function as antioxidants to reduce or terminate free radical chain reactions [20]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [21]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [22,23].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [24]. These various mechanisms underlie

several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [25,26]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that help to generate IL-1β is interesting to be explored in the future study.

APPENDICES

Acknowledgements

The authors are grateful to the LPPM Jenderal Soedirman University through Institution Research Grant (RISIN) No. T/324/UN23.18/PT.01.03/2020.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

Figures

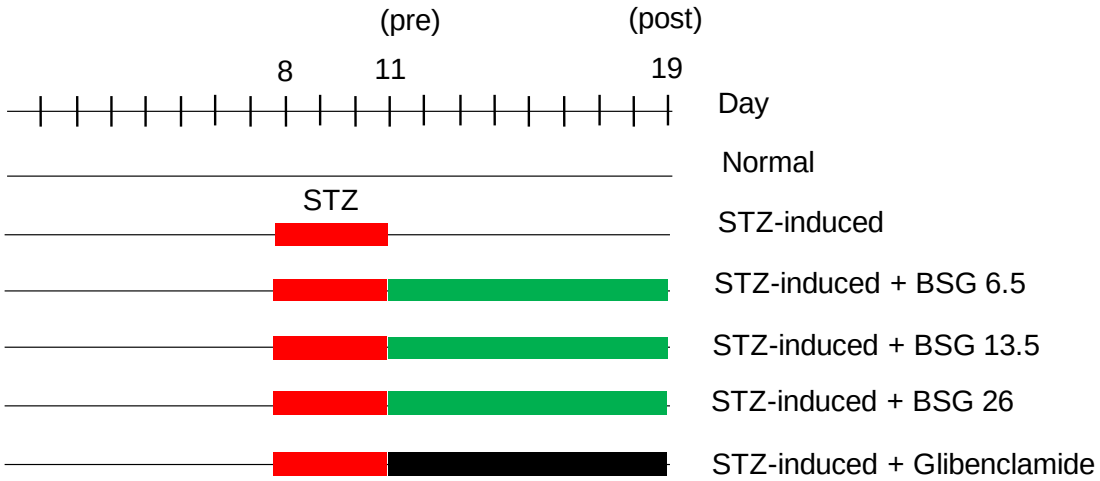

Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo garlic.

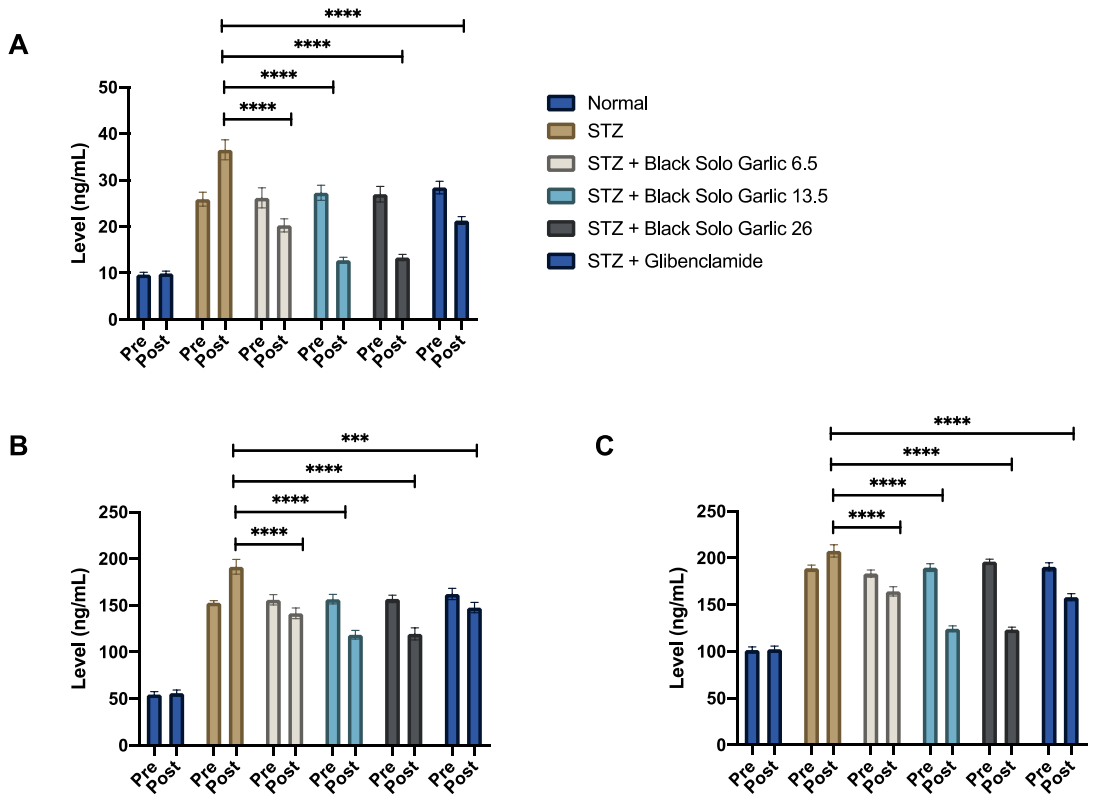

Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6, (B) IL-1β, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were

determined by ELISA. **** p <0,0001; *** p<0,0005

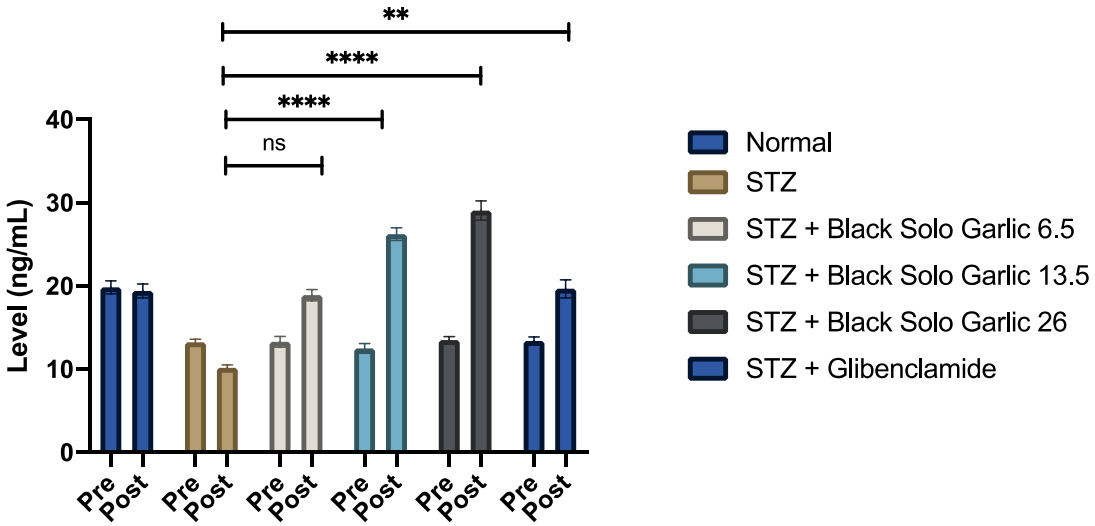

Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats was determined by ELISA. **** p<0,0001; ** p <0,01; ns = not significant.

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations (Sari et al., 2020). Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic (Tsalamandris et al., 2019).

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as single clove or solo garlic (*Allium sativum* L.), which contains unstable compounds such as allicin (Zhai et al., 2018). The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor (Chang, Jang, & Lin, 2021). Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects (Sembiring & Iskandar, 2019).

Black solo garlic contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity (Qiu, Zheng, Zhang, Sun-Waterhouse, & Qiao, 2020). Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation (Chang et al., 2021). The previous study showed that black ordinary garlic extract increases the immune system (Wang et al., 2010) and recover kidney cells (Lee et al., 2019). However, there has not been any study on immunomodulatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory effect of black solo garlic in streptozotocin (STZ)-induced rats.

2. Materials and methods

2.1. Preparation of steeping black solo garlic

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia. The selected garlics were approximately the same size and were fermented (aged) in the modified vehicle by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days.

After 21 days, the garlic turned into a black chewy texture, and were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 ° C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

2.2. Animals and Treatment

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used. The total number of animals was 30 rats, which were divided randomly into six group: normal, STZ-induced rat, STZ-induced rat treated with glibenclamide 0,09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of 6.5, 13.5, and 26 g/kg body weight.

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction, and after the induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19th (Figure 1). Also, steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.3. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits was used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol, using the Reader (Labotrone, Germany).

2.4. Statistical analysis

All data were presented as mean ± SEM and graphPad Prism 8 (California, US) were used to generate the figure. Furthermore, the mean scores between the treatment groups were compared with the one-way ANOVA and least significance different (LSD) test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level in comparison to the normal group (Figure 2). The administration of black solo garlic at dose of 13.5 g/kg and 26 g/kg reduced the level of IL-6, IL-1β, and TNF-α than STZ-induced only group. The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1β level more significant than those of glibenclamide (Figure 2B). Moreover, the treatment of black solo garlic was able to reduce TNF-α levels in all doses (Figure 2C). Therefore, the black solo garlic treatment dose of 13.5 g/kg equal with 26 g/kg body weight acts as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

In the treatment of STZ and black solo garlic in all doses, IFN-γ was reduced and increased respectively. Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the increased of IFN-γ levels than STZ-induced only group (Figure 3).

4. Discussion

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction (Graham, Janecek, Kittredge, Hering, & Schuurman, 2011), which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent (Thach, 2018). Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers (Nasr, 2014). During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling (Yang et al., 2017). Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

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reduced glutathione (GSH) and malondialdehyde (Naji, Al-Shaibani, Alhadi, Al-Soudi, & D'souza, 2017; Xiong, Jin, & You, 2018).

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α (You, Yoo, Baek, & Kim, 2019). These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ (Almatroodi et al., 2020; Saryono & Proverawati, 2019).

Conclusion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. Therefore, it can be used as an immunomodulator.

APPENDICES

Acknowledgements

The authors are grateful to the LPPM Jenderal Soedirman University through Institution Research Grant (RISIN) No. T/324/UN23.18/PT.01.03/2020.

Conflict of interest

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Authors' contributions

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Figures

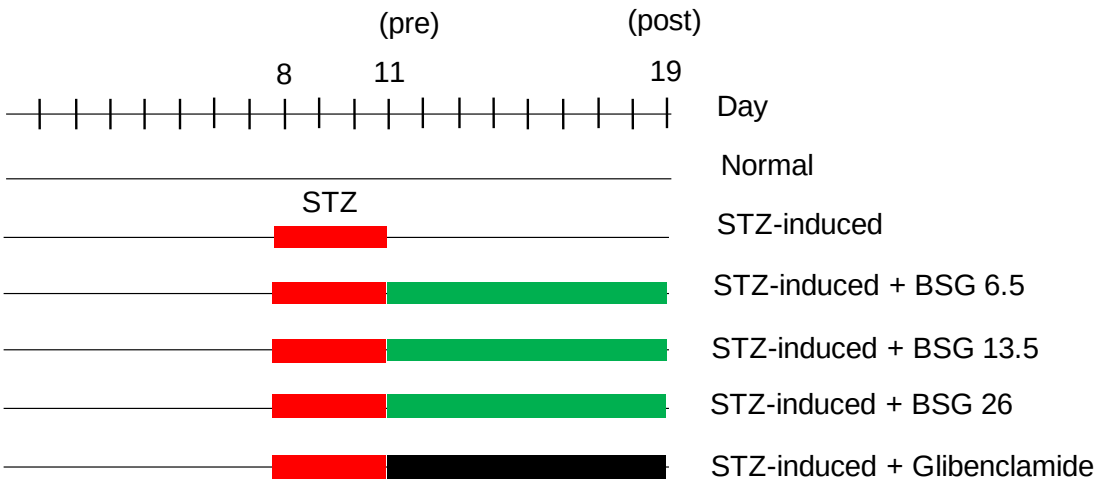

Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo

garlic.

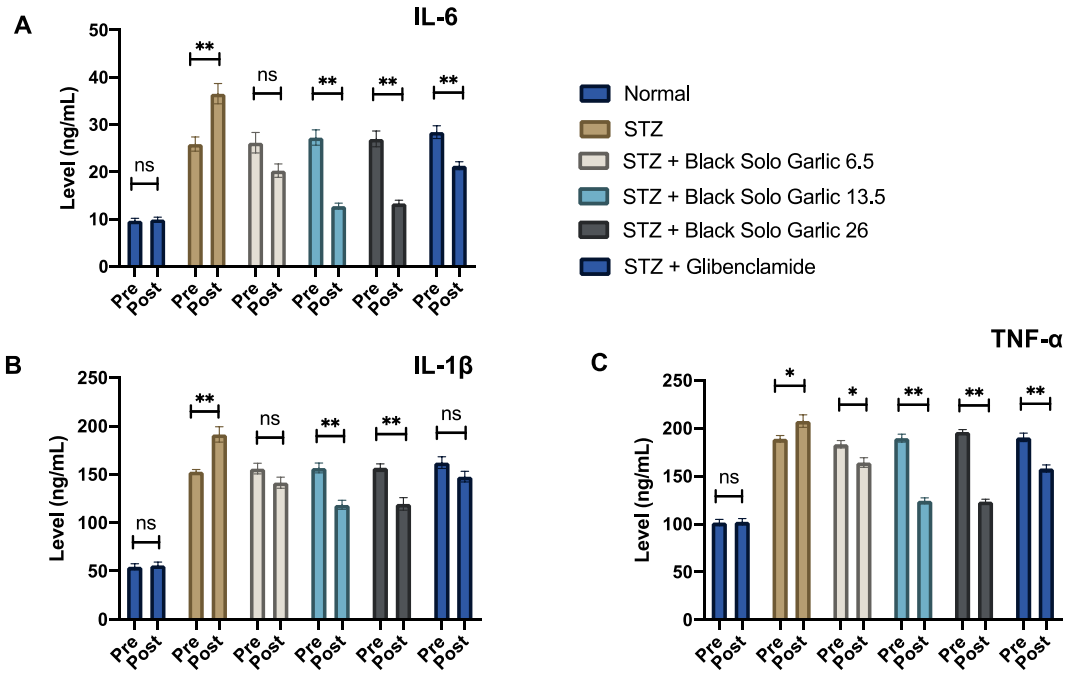

Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6 level, (B) IL-1β level, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were determined by ELISA. ** p <0,01; * p<0,05; ns = not significant.

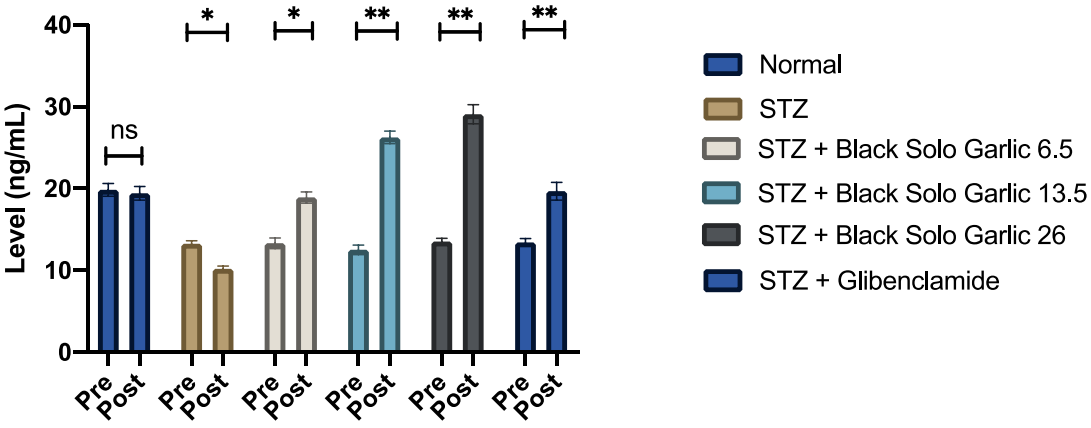

Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats was determined by ELISA. ** p <0,01; * p<0,05; ns = not significant.

Heliyon

Immunomodulatory effects of black solo garlic (Allium sativum L.) on streptozotocin-induced diabetes in Wistar rats

--Manuscript Draft--

Manuscript Number:	HELIYON-D-21-03591R2
Article Type:	Original Research Article
Keywords:	Black solo garlic; immunomodulator; Interleukin; interferon
Manuscript Classifications:	70: Food Science; 70.190: Food Chemistry; 110: Biological Sciences; 110.370: Diet; 130: Health Sciences; 130.280: Pharmacology; 130.500: Endocrinology; 130.500.110: Metabolism; 130.500.120: Nutrition
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Abstract:	Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1b (IL-1b) and interferon gamma (IFN-g) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1b, IL-6 and TNF-a as well as increased IFN-g with the immunity of STZ-induced rats.
Opposed Reviewers:	

Reviewer 1:

General comments:
Thank you for the effort on revising the manuscript, which is now look more comprehensive and understandable.
However, I still invite the revision from author for the following points:

1. Statistical analysis:
Thank you for revising the statistical analysis using ANOVA and comparing post-treatment results to the diabetic control group. I still have some comments for the statistical analysis comparison:
- Now, the figures lack statistical comparison between pre- and post-treatment results within the group. Please add the significant difference (if any) between pre- and post- treatment, for example using another symbol '#' and give an explanation in figure legend.

Response: We have revised the graph as reviewer suggested.

-In the result section, authors state:
"The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1 level more significant than those of glibenclamide"
"Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN- levels than those of STZ-induced only group."
However, still there is no direct statistical comparison (using asterisk mark) between black solo garlic group vs glibenclamide in the figure. Please add statistical difference marks between any group of treatment mentioned in the text (not only compared to control)

- One possible way to prevent the overcrowded figure with statistical significance marks is to build other figures with only data from post-treatment from each group, and use this figure to compare results from different groups of treatment. Please clarify this issue.

Response: We have revised the graph as reviewer suggested. However, we did not generate new figure; we added the comparison among groups in the same figure.

2. Identification of extract active content:
What was the result of GC-MS of fresh solo garlic and black solo garlic?
Please state the result either in method subchapter 'Plants' or in result section (with figure/table)

Response:

The extracts were further analysed by using GC-MS, and we found the same results as reported by Ref. 15.
This additional information was added in the Method section, subheading 2.3

3. Reviewer 2 points out an excellent point related to preliminary parameters, such as body weight, food and water intake and urine output as general indicators of diabetes. It is best if authors can present those parameters in the result section.

Response:

Food, water intake and urine output for animals were described in the Method section, subheading 2.4

4. Please pay attention to decimal separator. Sometimes author use 'point' as decimal separator (e.g.

BSG 13.5g/kg), while sometime authors use 'comma' (e.g. 0,09 mg/kg; p<0,0001). Please choose one decimal separator and use consistently throughout the text.

Response:
We corrected on the manuscript. Thank you.

Reviewer 3: Methods:
The used dose and the preparation of the extracts are not very clear.

It is not indicated if the extract is concentrated or dehydrated.

The dose of glibenclamide is very low and the one of the extracts she is very high, at least 10 mg/kg of glibenclamide and a maximum of 500 mg/kg for the extracts.

The objective of the work (line 67) mentions that the proinflammatory genes were evaluated, but those are not seen with Elisa

Response:
We added the information regarding how to obtain steeping of black solo garlic, in the Method section, subheading 2.6.
We did mistype in the manuscript regarding proinflammatory gene (line 67). In fact, what we mean is proinflammatory mediators.

Results:

Very limited, very little description of the figures.

Response:

We change the results part.

Manuscript Review

Manuscript General Indicators:

Publisher:	**Heliyon**
Manuscript Title:	**Immunomodulatory effects of black solo garlic (Allium sativum L.) on streptozotocin-induced diabetes in Wistar rats.**
Manuscript number:	**HELIYON-D-21-03591**

Authors:	**Saryono1, Desiyani Nani, Atikah Proverawati, Sarmoko**
Affiliation:	**University of Jenderal Soedirman, Dr. Soeparno Street Purwokerto 53123, Indonesia**

Reviewer's Comments:

Reviewer #1: The manuscript offers a new insight into the immunomodulatory activity of black garlic in diabetic animal models. However, some points need to be improved and/or clarified as follows:

Introduction

* *Why did authors measure IL-1, IL-6, TNF-α, and IFN- as the parameters in the immunomodulation of diabetic animal model? A reference for using those cytokines and chemokines will improve the introduction section.*

Response: We have added information about the relationship of inflammatory cytokines IL-1β, IL-6, TNF-α, as well as IFN-γ in diabetes (Ref 4,5) in the immunomodulation in Introduction.

Ref [4] Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57: 136–142. doi:10.1016/j.cyto.2011.09.029

[5] Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. J Inflamm Res. 2020;13: 187–193. doi:10.2147/JIR.S245064

Methods:

* *Line 59 → the modified vehicle → what is this modified vehicle?*

Response: vehicle means equipment that are needed for making black solo garlic. We change to "fermentation modified apparatus" refer to rice cooker.

* *Line 81 → steeping black solo garlic was given per oral → in what vehicle?*

Response: We did not use any vehicle because the solution is available as "steeping" form.

* *Section 2.2 → When was glibenclamide given (same schedule as extract?)? Further, it's not clear if the treatment was performed every day. Please clearly state this.*

Response: Yes, positive control was given in the same schedule with treatment group. Now we have added the information about schedule of glibenclamide on manuscript.

* *Section 2.4 → statistical analysis: using ANOVA? From the graph, it looks like each set of data (pre- and post-) was analyzed using paired t-test? If ANOVA was used, then authors also can compare the post-treatment data to the 'normal' group? Please clarify.*

Response: We re-analyzed for statistical analysis using Two-way ANOVA with a Tukey post-hoc test. Yes, we now compare the post-treatment data with other group data, including normal group. See the Figure 2 and 3 for new statistical analysis result.

Results and interpretation

* *Was there no identification of extract's active content? This will lead to a better discussion in the manuscript.*

Response: Yes, we did GC-MS to compare the content of fresh solo garlic and black solo. garlic.

* *Line 103: confusing sentence explaining the dose. Please rephrase*

Response: We modified to: “Therefore, treatment of the black solo garlic dose of 13.5 g/kg body weight are equal to those 26 g/kg body weight as an anti-inflammatory agent.”

* *Line 106-107: please rephrase the sentence. Better to divide into 2 sentence for better clarity*

Response: We split into two sentences as suggested: “Streptozotocin induction reduced IFN-gamma level in the rats. In contrast, black solo garlic in all doses significantly increased IFN-gamma level”

Related to statistical analysis (please also see last point in Method above)

* *Line 100: The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1 level more significant than those of glibenclamide → but there's no statistical analysis (in the figure) directly comparing two groups*

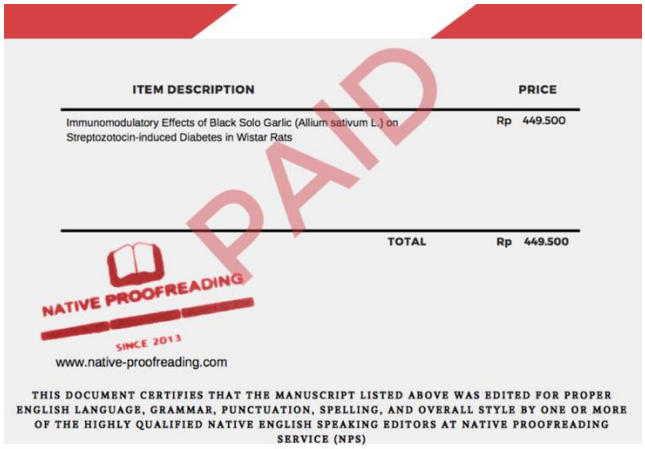
Response: We have added in the new Figure.

* *Line 108: Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the increased of IFN- levels than STZ-induced only group → but in figure, there's no direct statistical analysis between these groups.*

Response: We have added in the new Figure.

Lastly, the manuscript needs a professional English proofreader to improve the readability of the text.

Response: We have consulted to proofreading service.



Reviewer #2

I- Decision:

The article should be rejected as it needs major experimental and analytical work and revision of scientific information as per the stated comments.

II- General Comments:

Although, I do admire the good intentions of the authors for investigating the immunomodulatory actions of black solo garlic (BSG) in the model of STZ-Induced type-1 diabetes, nonetheless there is a lot that is missing to make this study credible and worth consideration for publication. I am sorry to say that the scientific argument presented, experimental and analytical work done, data presented and conclusion(s) reached do not mount to what is found even in a weak scientific poster presented by undergraduate students.

Response: Thank you and we have modified many parts as comments suggested. Although this study is quite simple, the study about black solo garlic (BSG) is important to add the effectiveness of BSG in inflammation field (in general) and diabetes (in specific).

III- Abstract:

The information presented in the abstract reflects the standard of the work done.

IV- Introduction: Is lacking major details and information.

In general, the scientific information presented in the introduction is superficial, inadequate and in certain places hardly relevant to and does not ideally set the scene for a logical and credible justification of why this study should be carried out (support the objective(s) and aim(s) of the study):

1. *The description of the condition of diabetes does not present accurate details of the subtypes.*

Response: Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin

deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required.

Ref. [3] Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. Int J Vasc Med. 2012;2012: 918267. doi:10.1155/2012/918267

2. *The casual connection made between the expected effects of treatment with BSG and the variables of inflammation leaves a lot to be desired. Also check the correct scientific name of solo garlic.*

Response:
Solo garlic known as single clove garlic, monobulb garlic, single bulb garlic, or pearl garlic is a type of *Allium sativum*. Scientific name of solo garlic: *Allium sativum* 'Solo garlic'. Diabetes is associated with high levels of cytokines TNF-alpha, IL-6, IL-1beta, and decreased interferon-gamma [4,5]. Single black garlic contains flavonoids as antioxidants and anti-inflammatory.

Ref [4] Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57: 136–142. doi:10.1016/j.cyto.2011.09.029

5. Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. J Inflamm Res. 2020;13: 187–193. doi:10.2147/JIR.S245064

3. *The reason(s) for choosing the selected (tested) inflammatory and anti-inflammatory variables (among many which have an effect on inflammatory responses) is not adequately stated. This point should be clarified and supported. Also the authors did not state the pleiotropic effects of the immune variables tested and how their modulations serves the objectives of the study.*

Response: Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ (Mirza et al. 2012; Kartika et al. 2020). TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production (Ortiz et al. 1995). IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy (Chang and Chuang 2010). IL-1 is a master cytokine of inflammation (Dinarello and van der Meer 2013).

Ref [4] Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57: 136–142. doi:10.1016/j.cyto.2011.09.029

5. Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. J Inflamm Res. 2020;13: 187–193. doi:10.2147/JIR.S245064
6. Ortiz A, Bustos C, Alonso J, Alcázar R, López-Armada MJ, Plaza JJ, et al. Involvement of tumor necrosis factor-alpha in the pathogenesis of experimental and human glomerulonephritis. Adv Nephrol Necker Hosp. 1995;24: 53–77.
7. Chang Y-C, Chuang L-M. The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication. Am J Transl Res. 2010;2: 316–331.

4. *A comparison between the content of aged regular garlic (multicloved) and BSG (single cloved) should be stated to highlight the significance of using extract of BSC in this study. Aged regular garlic also contains high amounts of remedial agents compared to fresh regular garlic.*

   Response: Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, SAC, and alkaloids content higher than those of fresh garlic [9]. black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14].

V- Objective(s) – Aim(s): Is lacking in details and information

A separate objective(s) – Aim(s) section is missing. If stating such sections is in tune with the journal guidelines for publication of articles, such section should be included.

Response: We added in the introduction.

VI- Methodology: Is lacking in details and information and more importantly significant steps and procedures.

1. *The methodology section needs to be divided into subsection for better presentation of the listed procedural steps.*

Response: Thank you. The new list has been made in the manuscript.

2. *The procedure describing the preparation – fermentation of fresh solo garlic to get BSG is vague. Was any liquid (such salted-water or vinegar, ethanol, etc.) or any other chemical added to fresh solo garlic to induced fermentation (or was it only dry fermentation)? Was a single batch of solo garlic fermented or more than one batch. If more than one batch was fermented, how the batches contents were standardized. Did the authors carry out GS-MS analysis to identify the different contents of this 'new' preparation to show its presumed and suggested active components content?*

Response: It's only dry fermentation. We used an 800 mL volume rice-cooker for fermentation so one batch fermentation is enough for sample's experiment. Yes, we did GC-MS to compare the content of fresh solo garlic and black solo garlic.

3. *What was the dose(s) of BSG that were administrated? What was the concentration of BSG in the seeping extract used for treatment?*

Response: We gave three doses for three different group: (i) 6.5, (ii) 13.5, and (iii) 26 g/kg body weight. BSG was mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 °C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

4. *How the efficacy of hyperglycaemia induction by STZ was tested to prove inflection of diabetes in the treated (and non-treated groups). Also, did the authors measure the concentration of glucose to ascertain the antidiabetic efficacy of BSG.*

Response: Yes, blood sugar was checked the second day after STZ induction.

5. *Did the authors measure the preliminary parameters (such as body weight, food and water intake and urine output – which are general indicators of diabetes) to show inflection of diabetes (in response to STZ) and recovery (in response to BSG)? Is the acute treatment with BSG (7-10 days) is enough to induce the effect of this preparation. Many variables were not measured and it is hard to ascertain this outcome. For positive control, the authors should have used more appropriate antidiabetic agents against type-1 (such as continuous insulin administration by mini-osmotic pumps or scheduled treatment with metformin). The use of glibenclamide for positive control is not appropriate as this antidiabetic drug is mostly used to treat type-2 diabetes. This drug stimulates the release of insulin from pancreatic beta-cells by depolarization. The suggestion that this drug is effective in the authors' model of STZ-induced (beta cell destruction) type 1 diabetes indicates that STZ was not effective. The measurement of insulin could have supported the arguments of the authors and the antidiabetic efficacy of their BSG preparation*

   Response: Yes, all parameters were recorded. Treatment of BSG for 8 days can suppress the inflammatory cytokines as shown in the Results. Those suggested methods such as continuous insulin administration by mini-osmotic pumps is difficult to be applied, and what we did already stated in the methods. Yes, we know the mechanism action of glibenclamide, and we want to see the protective effect glibenclamide in STZ-mice model. If the glibenclamide works, it does not mean the STZ-model was failed. Glibenclamide is commonly used in that model, for example in the https://journals.sagepub.com/doi/full/10.1177/1470320312460881 and https://www.sciencedirect.com/science/article/pii/S2211383512000263 and https://www.karger.com/Article/Fulltext/496104.

6. *The protocol of cytokines estimation needs further elucidation.*

   Response: We have added more detail information about this assay in the Method.

7. *As per the groups devised and treatments carried out a two-way ANOVA with a TuKey post-hoc test would have been more appropriate.*

Response: Thank you for your suggestion. We did statistical analysis with two-way ANOVA with a Tukey post-hoc test and the result shown in the Figure 2 and 3.

VII- Results: Is lacking in detail and superficial as a result of the simplicity of analysis carried out.

1. *Comparison between the groups is superficial and does not show the percentage changes in response to each experimental manoeuvre.*

   Response: Now we mentioned it in the text (Result section paragraph).

2. *The graphs included are redundant.*

   Response: A new Figure has been drawn that show significant between STZ-induced group with treatment group. (Figure 2 and 3)

VIII- Discussion: is lacking in details and information.

1. *The stated information and level of argument is superficial, disjointed and even loosely related. Listing scientific information just for the sake of it does not support and provide merited and well-founded discussion.*

   Response: We re-arrange the discussion part.

2. *The argued mechanisms of increase (due to the induction of diabetes) and decrease (due to treatment with BSG) in inflammatory and anti-inflammatory variables is flimsy.*

   Response: We re-arrange the discussion part.

IX- Conclusions:

The couple of statements listed can hardly be considered as a conclusion. They are no more than restating what was already mentioned in the result section.

Response: We delete this section and elaborate in the discussion part.

Bibliography

Chang, Y.-C. and Chuang, L.-M. 2010. The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication. *American journal of translational research* 2(3), pp. 316–331.

Dinarello, C.A. and van der Meer, J.W.M. 2013. Treating inflammation by blocking interleukin-1 in humans. *Seminars in Immunology* 25(6), pp. 469–484.

Kartika, R., Purnamasari, D., Pradipta, S., Larasati, R.A. and Wibowo, H. 2020. Impact of Low Interferon-γ and IL-10 Levels on TNF-α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus. *Journal of inflammation research* 13, pp. 187–193.

Mirza, S., Hossain, M., Mathews, C., Martinez, P., Pino, P., Gay, J.L., Rentfro, A., McCormick, J.B. and Fisher-Hoch, S.P. 2012. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. *Cytokine* 57(1), pp. 136–142.

Ortiz, A., Bustos, C., Alonso, J., Alcázar, R., López-Armada, M.J., Plaza, J.J., González, E. and Egido, J. 1995. Involvement of tumor necrosis factor-alpha in the pathogenesis of experimental and human glomerulonephritis. *Advances in nephrology from the Necker Hospital* 24, pp. 53–77.

Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). Fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic'), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory mediators (TNF-α, IL-6, IL-1β, IFN-γ) to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days. After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (at a temperature of 80-90 °C) was added and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic. The steeping were further analysed by using GC-MS, and we found the same results as reported by Tran et al. (2018) [15].

2.4. Animals

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used. They were given *ad libitum* access to standard food and distilled water.

2.5 Streptozotocin-induced diabetic rats

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0.09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5 (low dose), (v) 13.5 (medium dose), and (vi) 26 g/kg body weight (high dose). After STZ-induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19$^{th.}$ The same schedule was also applied for glibenclamide treatment (Figure 1). Briefly, the black solo garlic powder was

measured based on the bodyweight of rats to obtain those three doses. The measured powder was diluted in the 3.6 mL hot water and stirred for 15 minutes to obtain steeping of black solo garlic. Steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits were used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. Briefly, this ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

2.8. Statistical analysis

All data were presented as mean ± SEM. GraphPad Prism 8 (California, US) was used to generate the figure. Furthermore, the mean scores among the treatment groups were compared with the one-way ANOVA with a Tukey post-hoc test. Pre- and post-test treatment were analyzed by t-test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level compared normal group ($p<0.0001$) (Figure 2). The administration of black solo garlic at dose of 6.5 g/kg, 13.5 g/kg and 26 g/kg body weight had significantly reduced the level of IL-1β, IL-6, and TNF-α compared to untreated diabetic rats ($p<0.0001$). Interestingly, the treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to reduce IL-1β, IL-6, and TNF-α level lower than those of glibenclamide. The effects on IL-1β, IL-6, and TNF-α were dose dependent, the reduced level of those cytokines in rats administrated with the medium and high black solo garlic dose (13.5 and 26 g/kg, respectively) being significantly greater than that in the rats receiving low black solo garlic dose (6.5 mg/kg). Therefore, treatment of the black solo garlic dose of 13.5 g/kg are equal to those 26 g/kg body weight as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction produced a significant decreased of IFN-γ compared to the normal group ($p<0.0001$) (Figure 3). Black solo garlic (13.5 and 26 g/kg)-treated diabetic rats had significantly increased level IFN-γ compared to the untreated diabetic rats ($p<0.0001$). The effects on IFN-γ were dose dependent, IFN-γ levels of rats having been administrated medium or high black solo garlic dose being significantly higher than those in the diabetic rats receiving the low black solo garlic dose. Moreover, the treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to increase IFN-γ level higher than those of glibenclamide.

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [16], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [17]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [18]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling [19]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [20]. Flavonoids can function as antioxidants to reduce or terminate free

radical chain reactions [21]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [22]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [23,24].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [25]. These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [26,27]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that have critical role in generating IL-1β is interesting to be explored in the future study.

APPENDICES

Acknowledgements

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Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

Figures

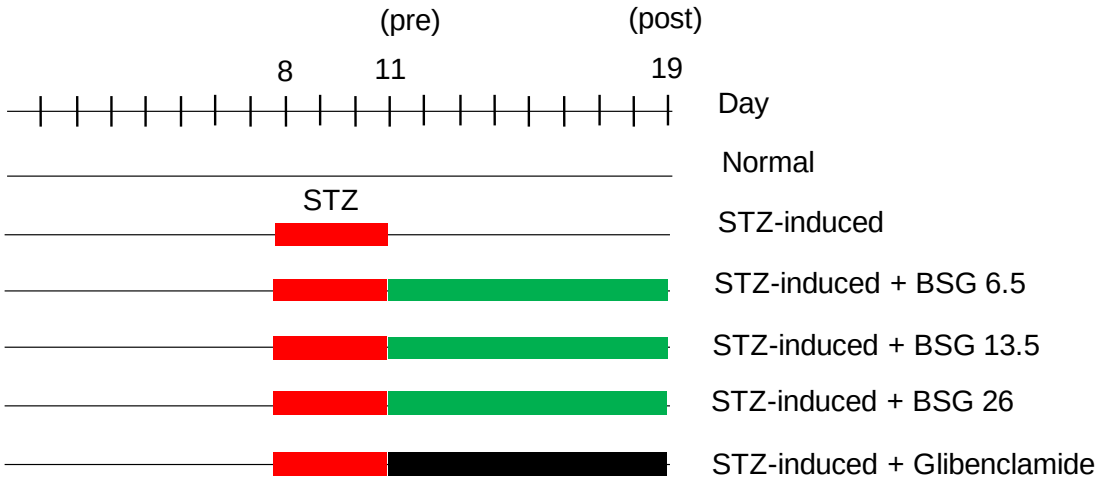

Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo garlic.

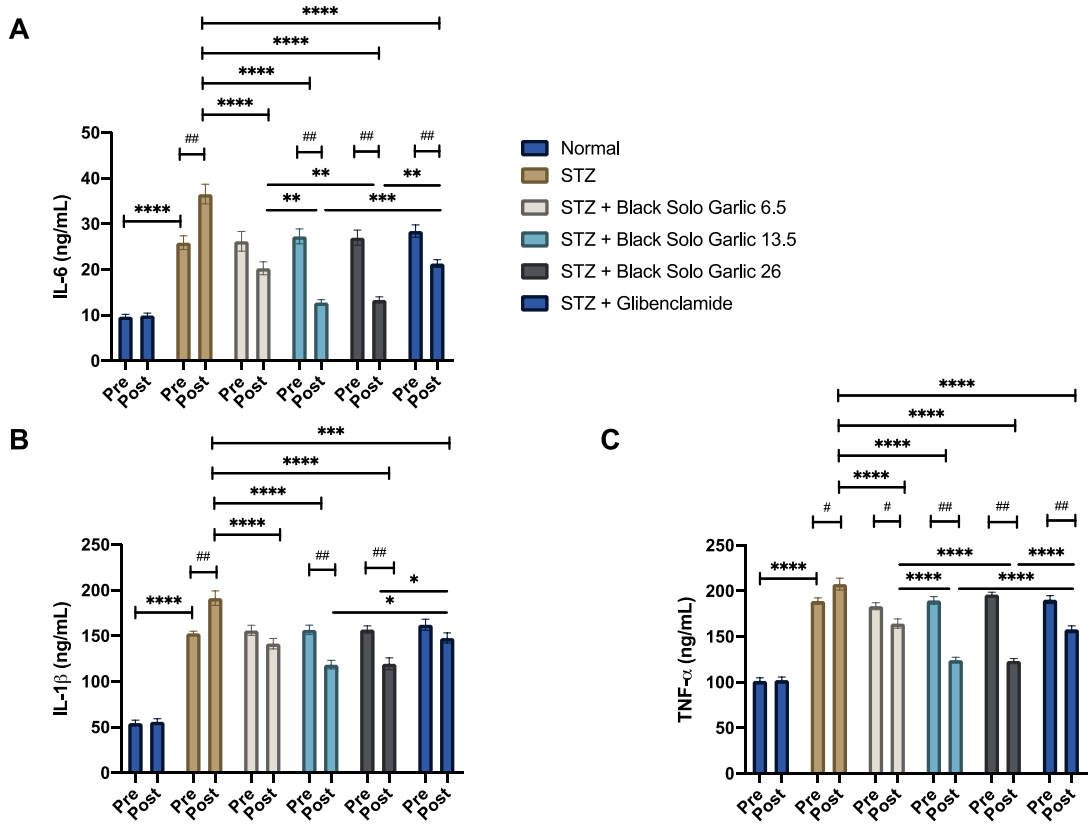

Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6, (B) IL-1β, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26

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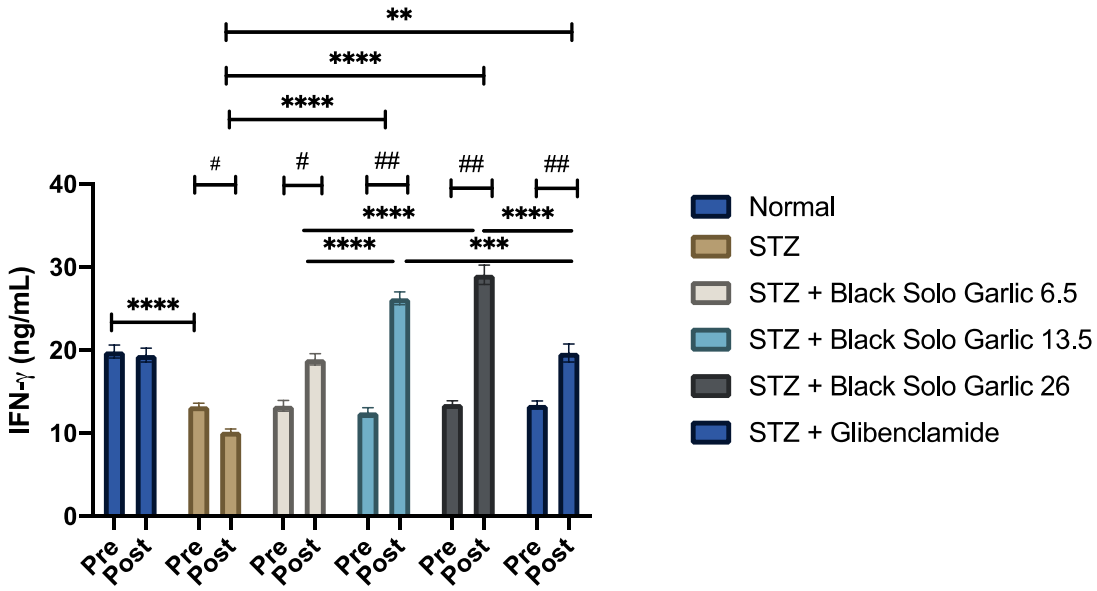

Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0.09 mg/kg in streptozotocin-induced rats was determined by ELISA. Statistical significance for the difference among groups: **** p<0.0001; *** p<0.0005; ** p <0.01. Statistical significance for the difference between the data of pre-test group vs post-test groups: ## p<0.01; # p<0.05

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). Fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic'), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory ~~genes~~ mediators (TNF-α, IL-6, IL-1β, IFN-γ) ~~expression~~ to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days. After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (at a temperature of 80-90 °C~~200 ml per dose~~) was added ~~at a temperature of 80-90 °C~~ and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic ~~extract~~. The steeping were further analysed by using GC-MS, and we found the same results as reported by Tran et al. (2018) [15].

2.4. Animals

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used. They were given *ad libitum* access to standard food and distilled water.

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2.5 Streptozotocin-induced diabetic rats

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0.~~,~~09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5 (low dose), (v) 13.5 (medium dose), and (vi) 26 g/kg body weight (high dose). After STZ~~the~~ induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19$^{th.}$ The same schedule was also applied

for glibenclamide treatment (Figure 1). Briefly, the black solo garlic powder was measured based on the bodyweight of rats to obtain those three doses. The measured powder was diluted in the 3.6 mL hot water and stirred for 15 minutes to obtain steeping of black solo garlic. Steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits were used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. Briefly, this ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

2.8. Statistical analysis

All data were presented as mean ± SEM. ~~and~~ GraphPad Prism 8 (California, US) was used to generate the figure. Furthermore, the mean scores among~~between~~ the treatment groups were compared with the two-way ANOVA with a Tukey post-hoc test. Pre- and post-test treatment were analyzed by t-test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level ~~in comparison~~compared ~~to the normal~~ normal group ($p<0.0001$)~~group~~ (Figure 2). The administration of black solo garlic at dose of 6.5 g/kg, 13.5 g/kg and 26 g/kg body weight had significantly reduced ~~50%~~ the level of IL-1β, IL-6, and ~~reduced 37% the level of IL-1β, and~~ TNF-α ~~-~~compared to~~than STZ-induced only group~~untreated diabetic rats ($p<0.0001$). Interestingly, t~~T~~he treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to reduce IL-1β, IL-6, and TNF-α ~~-~~level lower ~~more significant~~ than those of glibenclamide ~~(Figure 2B)~~. The effects on IL-1β, IL-6, and TNF-α were dose dependent, the reduced level of those cytokines in rats administrated with the medium and high black solo garlic dose (13.5 and 26 g/kg, respectively) being significantly greater than that in the rats receiving low black solo garlic dose (6.5 mg/kg). ~~Moreover, the treatment of black solo garlic was able to reduce TNF-α levels~~

~~in all doses (Figure 2C).~~ Therefore, treatment of the black solo garlic dose of 13.5 g/kg ~~body weight~~ are equal to those 26 g/kg body weight as an anti-inflammatory agent.

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3.2. The effect of steeping black solo garlic on IFN-γ

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~~Streptozotocin induction reduced IFN-γ level in the rats. In contrast, black solo garlic in all doses increased IFN-γ level (Figure 3). Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN-γ levels than those of STZ-induced only group.~~

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

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APPENDICES

Acknowledgements

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Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

Figures

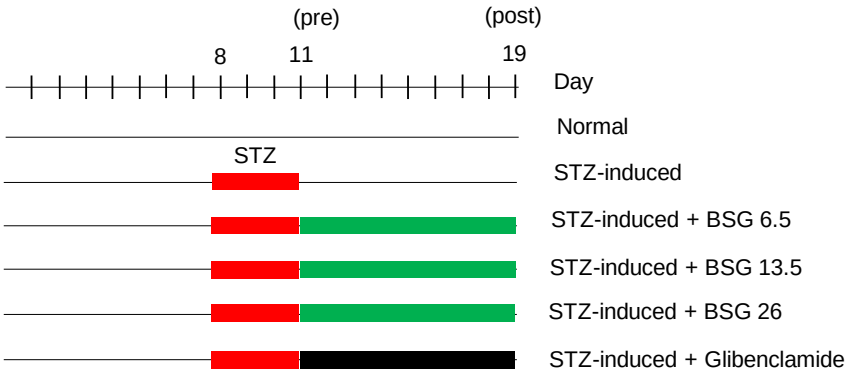
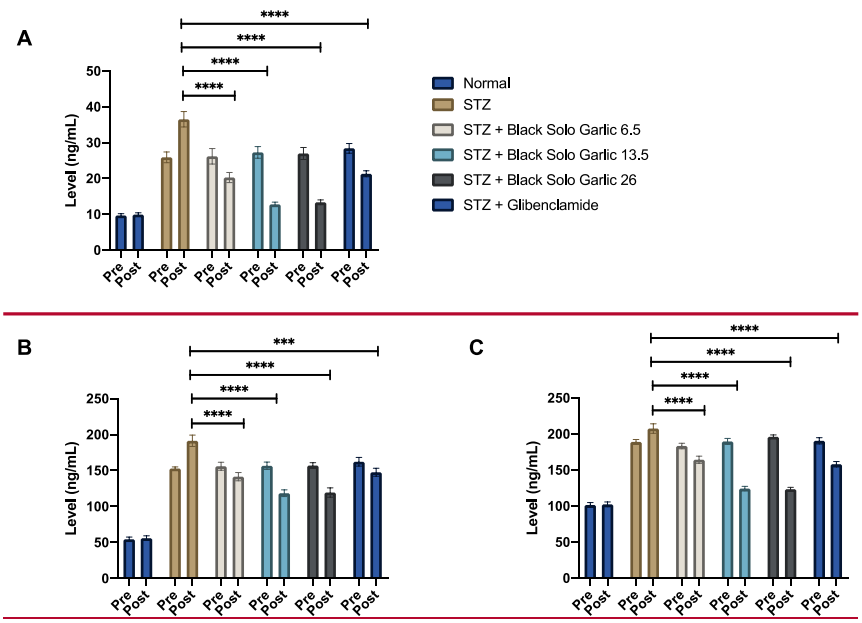


Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo garlic.



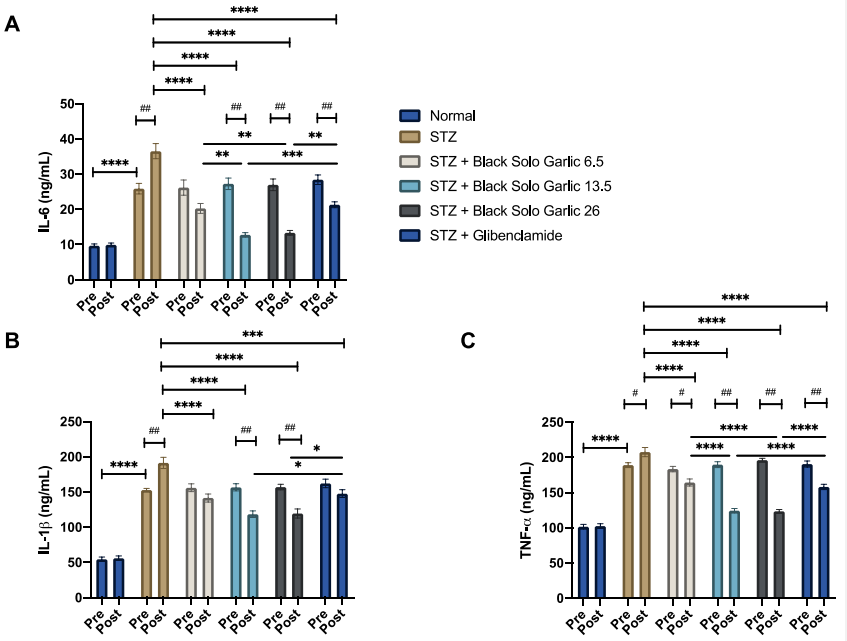


Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6, (B) IL-1β, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were determined by ELISA. Statistical significance for the difference among groups: **** p<0.0001; *** p<0.0005; *p<0.05. Statistical significance for the difference between the data of pre-test group vs post-test groups: ## p<0.01; # p<0.05

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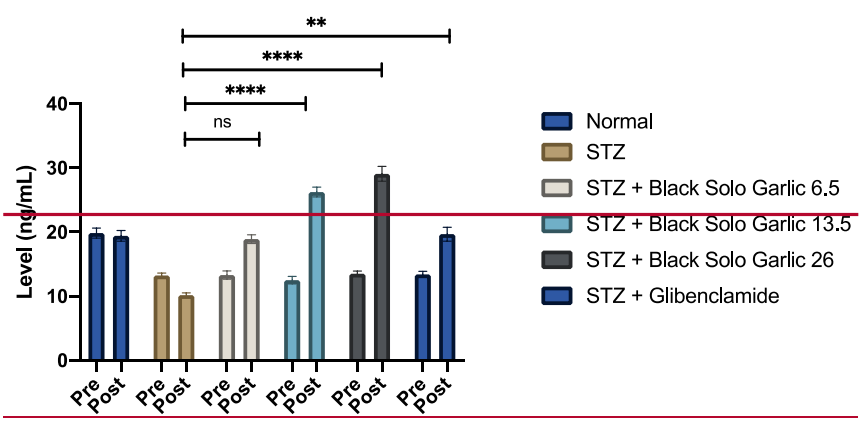

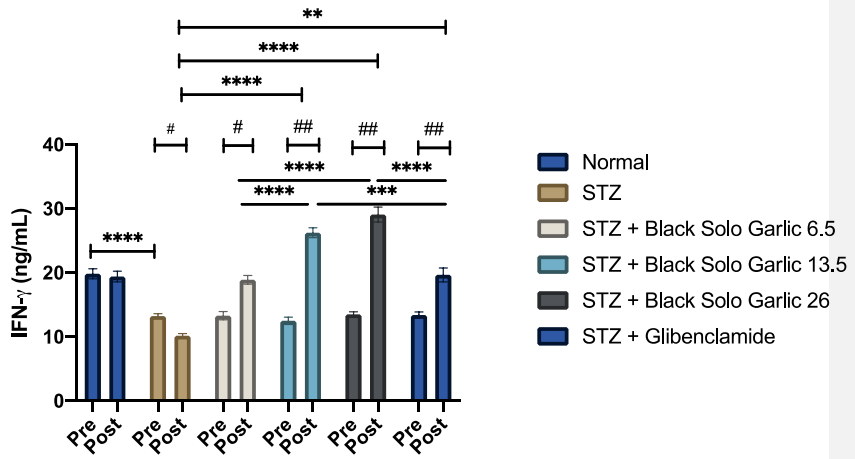

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). Fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic'), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory genes (TNF-α, IL-6, IL-1β, IFN-γ) expression to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days. After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 °C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

2.4. Animals

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used.

2.5 Streptozotocin-induced diabetic rats

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0,09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5, (v) 13.5, and (vi) 26 g/kg body weight. After the induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19$^{th.}$ The same schedule was also applied for glibenclamide treatment (Figure 1). Steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits were used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. Briefly, this ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

2.8. Statistical analysis

All data were presented as mean ± SEM and GraphPad Prism 8 (California, US) was used to generate the figure. Furthermore, the mean scores between the treatment groups were compared with the two-way ANOVA with a Tukey post-hoc test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level in comparison to the normal group (Figure 2). The administration of black solo garlic at dose of 13.5 g/kg and 26 g/kg reduced 50% the level of IL-6 and reduced 37% the level of IL-1β, and TNF-α than STZ-induced only group. The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1β level more significant than those of glibenclamide (Figure 2B). Moreover, the treatment of black solo garlic was able to reduce TNF-α levels in all doses (Figure 2C). Therefore, treatment of the black solo garlic dose of 13.5 g/kg body weight are equal to those 26 g/kg body weight as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction reduced IFN-γ level in the rats. In contrast, black solo garlic in all doses increased IFN-γ level (Figure 3). Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN-γ levels than those of STZ-induced only group.

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [15], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [16]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [17]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling [18]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [19]. Flavonoids can function as antioxidants to reduce or terminate free radical chain reactions [20]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [21]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [22,23].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [24]. These various mechanisms underlie

several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [25,26]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that help to generate IL-1β is interesting to be explored in the future study.

APPENDICES

Acknowledgements

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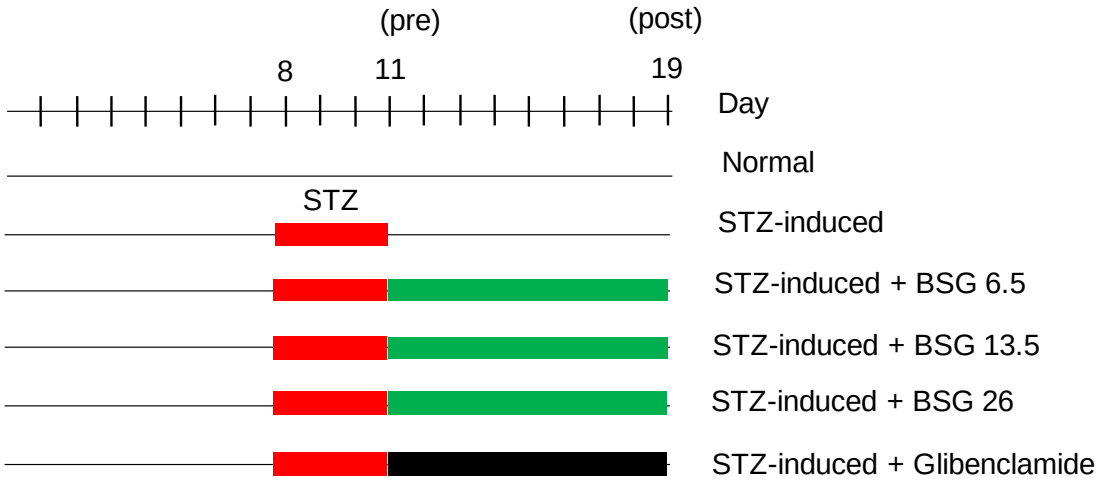
Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

Figures



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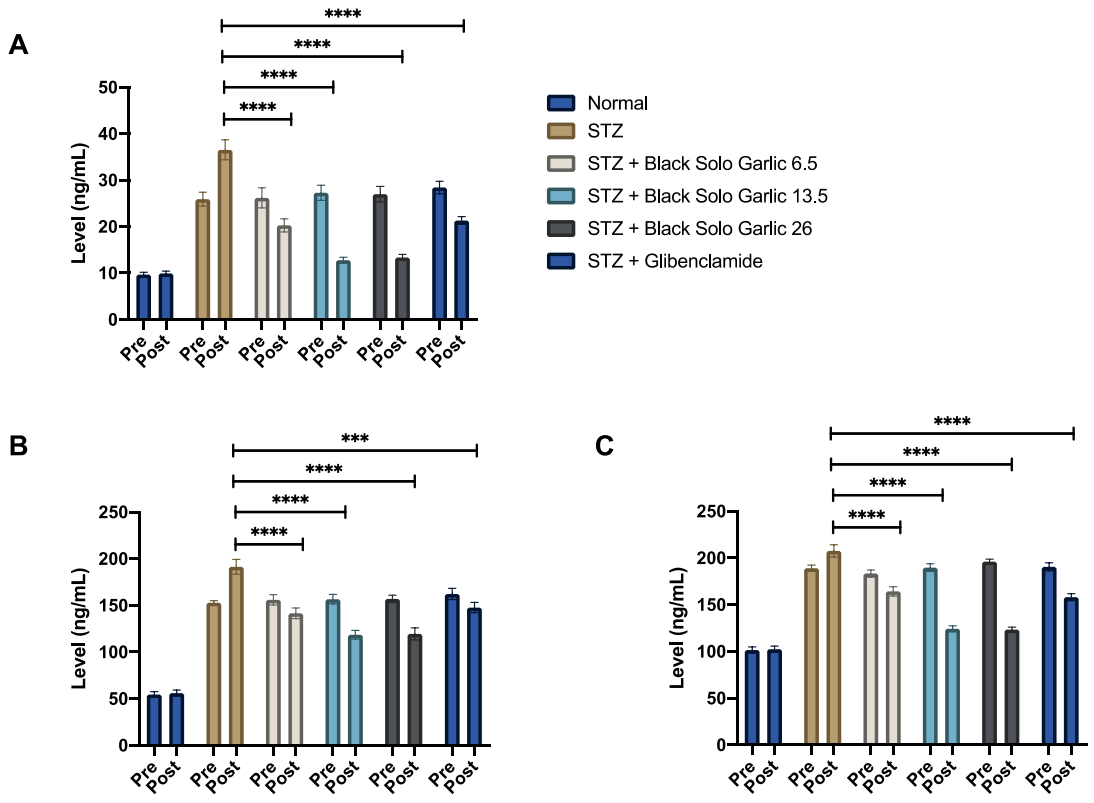

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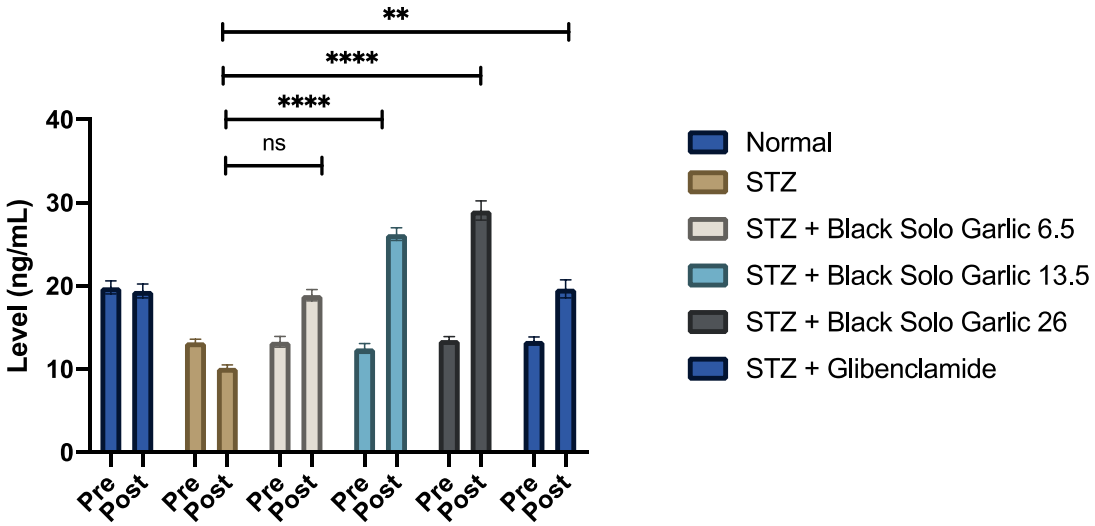

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). F~~f~~ermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. ~~Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [5].~~Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic' ~~L.~~), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, ~~fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [4]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [5].~~

b~~B~~lack solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. ~~{...}[7]{...}The previous study showed that black ordinary garlic extract increases the immune system [8] and recover kidney cells [9].~~ However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore,

this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory genes (TNF-α, IL-6, IL-1β, IFN-γ) expression to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

~~**2.1. reparation of steeping black solo garlic**~~

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus~~vehicle~~ by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days.

After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic~~, and~~ were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 °-C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

~~2.2.~~ 2.4. Animals ~~and Treatment~~

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used.

2.5 Streptozotocin-induced diabetic rats

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

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2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0,09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5, (v) 13.5, and (vi) 26 g/kg body weight.

~~Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction~~A~~, and a~~fter the induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19th. The same schedule was also applied for glibenclamide treatment (Figure 1). S~~Also, s~~teeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

~~2.3.~~ 2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits~~s~~ were~~as~~ used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. ~~, using the Reader (Labotrone, Germany)~~Briefly, t~~,~~his ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

~~2.4.~~ 2.8. Statistical analysis

All data were presented as mean ± SEM and G~~g~~raphPad Prism 8 (California, US) was~~ere~~ used to generate the figure. Furthermore, the mean scores between the treatment groups were compared with the two~~one~~-way ANOVA with a Tukey post-hoc test~~and least significance different (LSD) test~~. p<0.05 was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level in comparison to the normal group (Figure 2). The administration of black solo garlic at dose of 13.5 g/kg and 26 g/kg reduced 50% the level of IL-6~~,~~ and reduced 37% the level of IL-1β, and TNF-α ~~IL-1β, and TNF-α~~ than STZ-induced only group. The

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treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1β level more significant than those of glibenclamide (Figure 2B). Moreover, the treatment of black solo garlic was able to reduce TNF-α levels in all doses (Figure 2C). Therefore, treatment of the black solo garlic ~~treatment~~ dose of 13.5 g/kg body weight are equal ~~with~~ to those 26 g/kg body weight ~~acts~~ as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction reduced IFN-γ level in the rats. In contrast, ~~In the treatment of STZ and~~ black solo garlic in all doses increased~~,~~ IFN-γ ~~was reduced and increased respectively~~level (Figure 3). Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN-γ levels than those of STZ-induced only group~~p~~ ~~(Figure 3)~~.

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [15], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [16]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [17]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling [18]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [19]. Flavonoids can function as antioxidants to reduce or terminate free radical chain reactions [20]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [21]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [22,23].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [24]. These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [25,26]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that help to generate IL-1β is interesting to be explored in the future study.

~~**Conclusion**~~

~~The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. Therefore, it can be used as an immunomodulator.~~

APPENDICES

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

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Figures

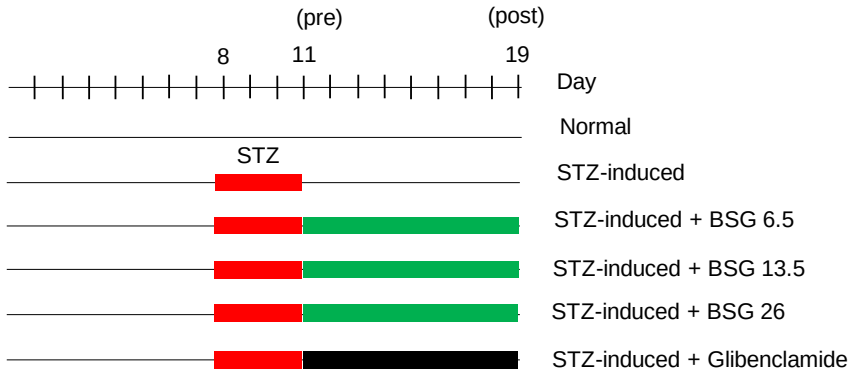

Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo

garlic.

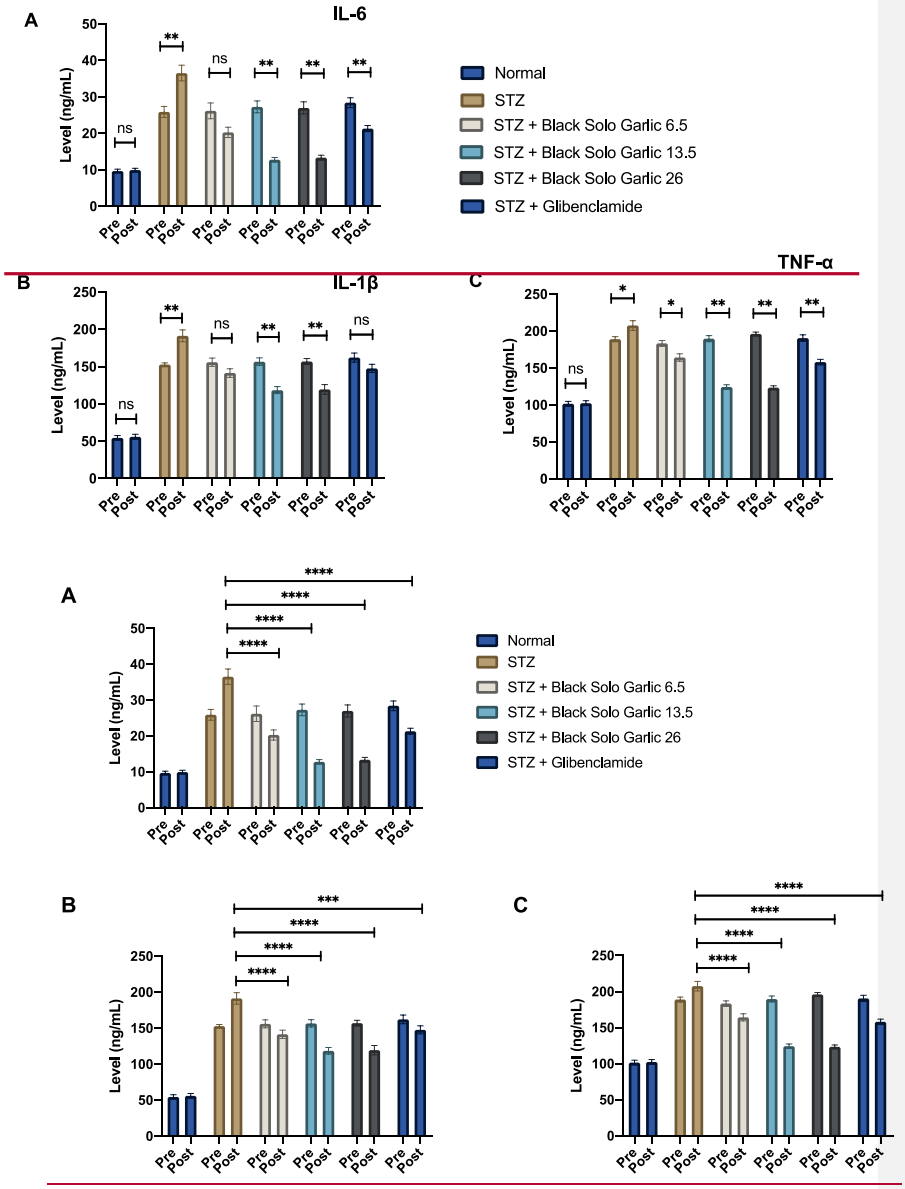

Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6 ~~level~~, (B) IL-1β ~~level~~, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were determined by ELISA. ** p <0,0001; * p<0,00055; ~~ns = not~~

~~significant.~~

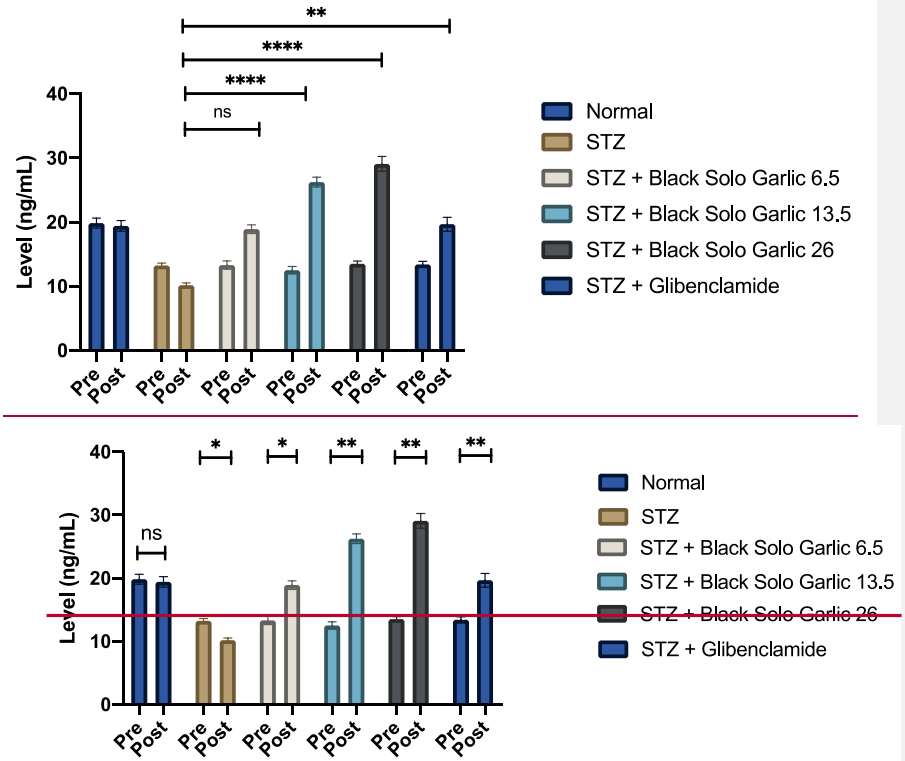

Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats was determined by ELISA. **** p<0,0001; ** p <0,01; ~~* p<0,05;~~ ns = not significant.

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations (Sari et al., 2020). Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic (Tsalamandris et al., 2019).

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as single clove or solo garlic (*Allium sativum* L.), which contains unstable compounds such as allicin (Zhai et al., 2018). The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor (Chang, Jang, & Lin, 2021). Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects (Sembiring & Iskandar, 2019).

Black solo garlic contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity (Qiu, Zheng, Zhang, Sun-Waterhouse, & Qiao, 2020). Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation (Chang et al., 2021). The previous study showed that black ordinary garlic extract increases the immune system (Wang et al., 2010) and recover kidney cells (Lee et al., 2019). However, there has not been any study on immunomodulatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory effect of black solo garlic in streptozotocin (STZ)-induced rats.

2. Materials and methods

2.1. Preparation of steeping black solo garlic

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia. The selected garlics were approximately the same size and were fermented (aged) in the modified vehicle by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days.

After 21 days, the garlic turned into a black chewy texture, and were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (200 ml per dose) was added at a temperature of 80-90 ° C and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic extract.

2.2. Animals and Treatment

The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used. The total number of animals was 30 rats, which were divided randomly into six group: normal, STZ-induced rat, STZ-induced rat treated with glibenclamide 0,09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of 6.5, 13.5, and 26 g/kg body weight.

Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction, and after the induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19th (Figure 1). Also, steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.3. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits was used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol, using the Reader (Labotrone, Germany).

2.4. Statistical analysis

All data were presented as mean ± SEM and graphPad Prism 8 (California, US) were used to generate the figure. Furthermore, the mean scores between the treatment groups were compared with the one-way ANOVA and least significance different (LSD) test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level in comparison to the normal group (Figure 2). The administration of black solo garlic at dose of 13.5 g/kg and 26 g/kg reduced the level of IL-6, IL-1β, and TNF-α than STZ-induced only group. The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1β level more significant than those of glibenclamide (Figure 2B). Moreover, the treatment of black solo garlic was able to reduce TNF-α levels in all doses (Figure 2C). Therefore, the black solo garlic treatment dose of 13.5 g/kg equal with 26 g/kg body weight acts as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

In the treatment of STZ and black solo garlic in all doses, IFN-γ was reduced and increased respectively. Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the increased of IFN-γ levels than STZ-induced only group (Figure 3).

4. Discussion

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction (Graham, Janecek, Kittredge, Hering, & Schuurman, 2011), which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent (Thach, 2018). Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers (Nasr, 2014). During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling (Yang et al., 2017). Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals (Ryu & Kang, 2017). Flavonoids can function as antioxidants to reduce or terminate free radical chain reactions (Lu, Li, Qiao, Qiu, & Liu, 2017). *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species (Tsai et al., 2019). In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase,

reduced glutathione (GSH) and malondialdehyde (Naji, Al-Shaibani, Alhadi, Al-Soudi, & D'souza, 2017; Xiong, Jin, & You, 2018).

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α (You, Yoo, Baek, & Kim, 2019). These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ (Almatroodi et al., 2020; Saryono & Proverawati, 2019).

Conclusion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. Therefore, it can be used as an immunomodulator.

APPENDICES

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing

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Figures

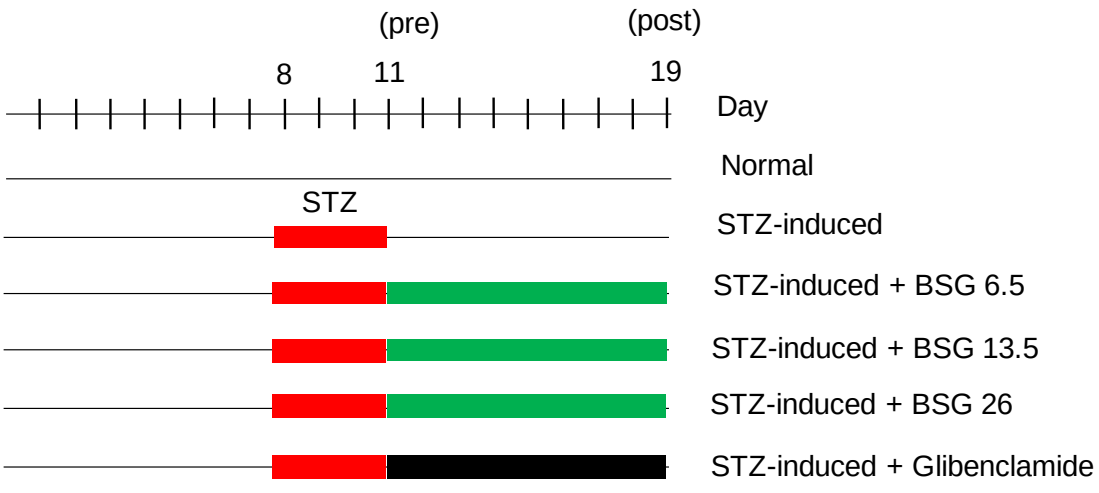


Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo

garlic.

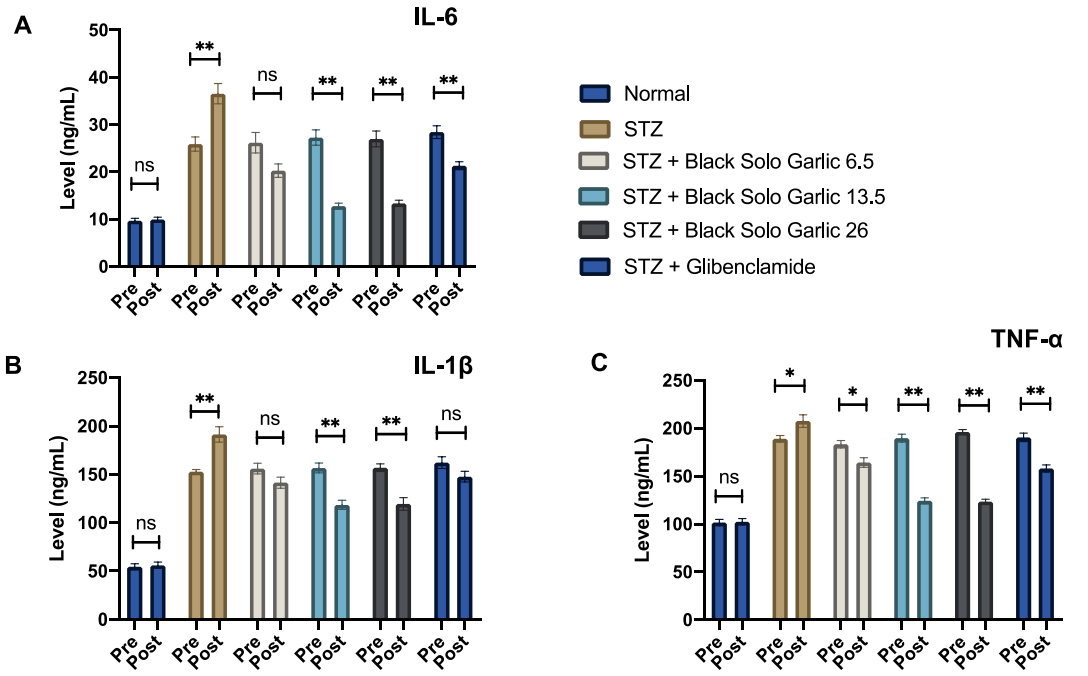


Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6 level, (B) IL-1β level, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were determined by ELISA. ** p <0,01; * p<0,05; ns = not significant.

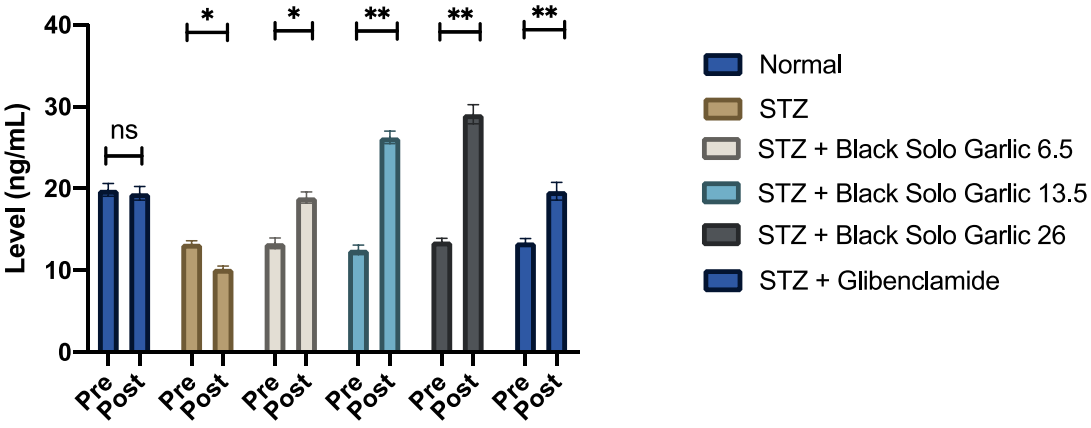
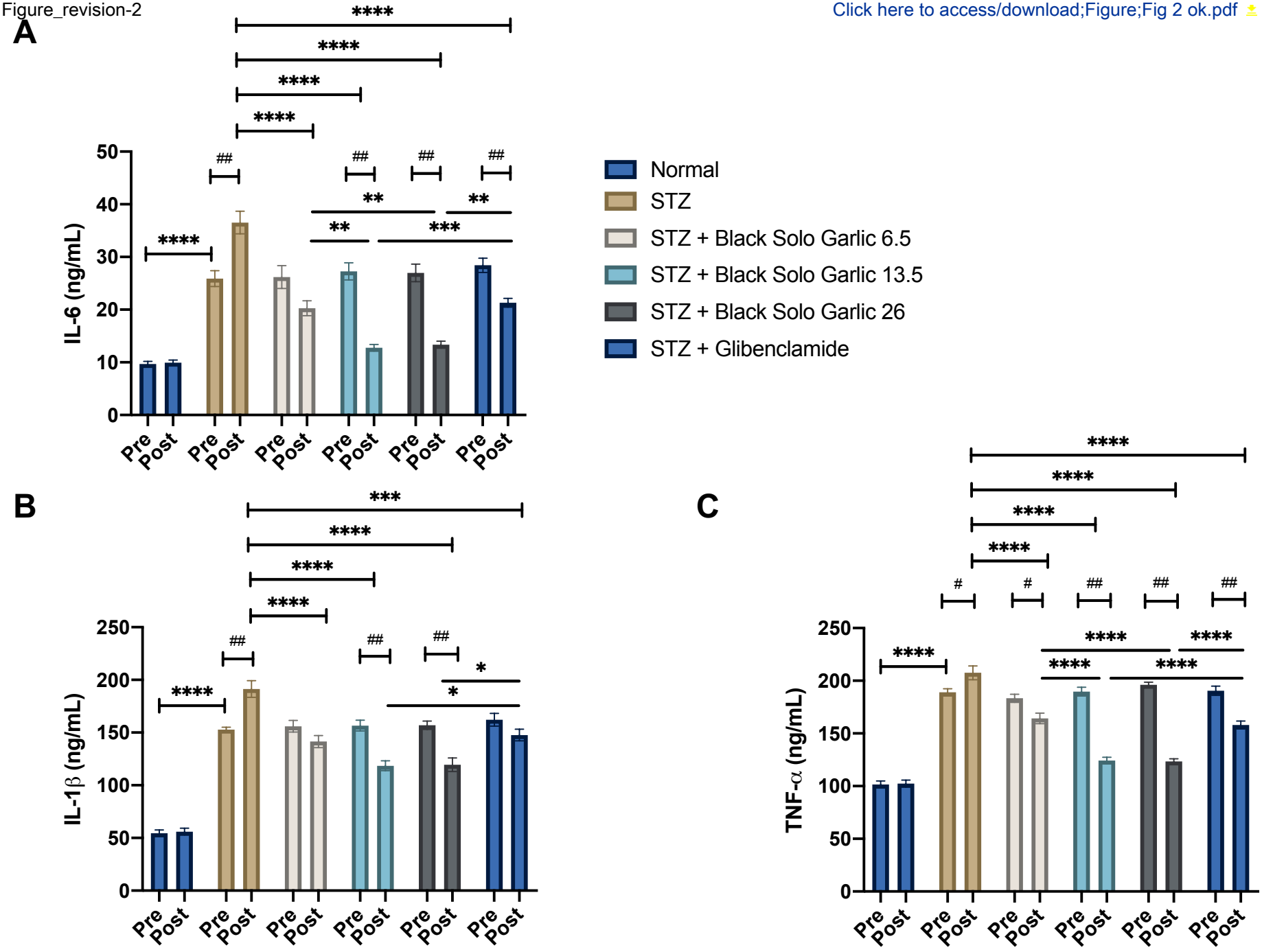


Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats was determined by ELISA. ** p <0,01; * p<0,05; ns = not significant.



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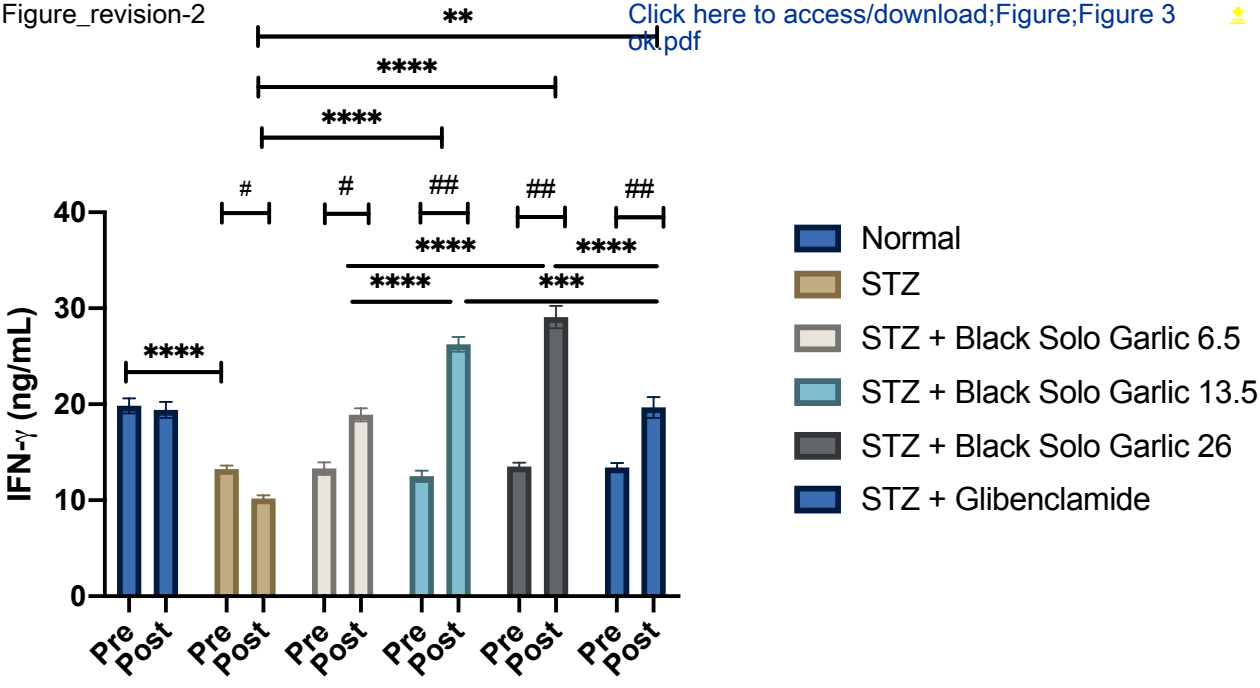

Heliyon

Immunomodulatory effects of black solo garlic (Allium sativum L.) on streptozotocin-induced diabetes in Wistar rats

--Manuscript Draft--

Manuscript Number:	HELIYON-D-21-03591R3
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Manuscript Classifications:	70: Food Science; 70.190: Food Chemistry; 110: Biological Sciences; 110.370: Diet; 130: Health Sciences; 130.280: Pharmacology; 130.500: Endocrinology; 130.500.110: Metabolism; 130.500.120: Nutrition
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First Author:	Saryono Saryono, Dr.
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	Desiyani Nani
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Abstract:	Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1b (IL-1b) and interferon gamma (IFN-g) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1b, IL-6 and TNF-a as well as increased IFN-g with the immunity of STZ-induced rats.
Opposed Reviewers:	

Reviewer 1:

General comments:
Thank you for the effort on revising the manuscript, which is now look more comprehensive and understandable.
However, I still invite the revision from author for the following points:

1. Statistical analysis:
Thank you for revising the statistical analysis using ANOVA and comparing post-treatment results to the diabetic control group. I still have some comments for the statistical analysis comparison:
- Now, the figures lack statistical comparison between pre- and post-treatment results within the group. Please add the significant difference (if any) between pre- and post- treatment, for example using another symbol '#' and give an explanation in figure legend.

Response: We have revised the graph as reviewer suggested.

-In the result section, authors state:
"The treatment at dose of 13.5 g/kg and 26 g/kg was able to reduce IL-1 level more significant than those of glibenclamide"
"Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN- levels than those of STZ-induced only group."
However, still there is no direct statistical comparison (using asterisk mark) between black solo garlic group vs glibenclamide in the figure. Please add statistical difference marks between any group of treatment mentioned in the text (not only compared to control)

- One possible way to prevent the overcrowded figure with statistical significance marks is to build other figures with only data from post-treatment from each group, and use this figure to compare results from different groups of treatment. Please clarify this issue.

Response: We have revised the graph as reviewer suggested. However, we did not generate new figure; we added the comparison among groups in the same figure.

2. Identification of extract active content:
What was the result of GC-MS of fresh solo garlic and black solo garlic?
Please state the result either in method subchapter 'Plants' or in result section (with figure/table)

Response:

The extracts were further analysed by using GC-MS, and we found the same results as reported by Ref. 15.
This additional information was added in the Method section, subheading 2.3

3. Reviewer 2 points out an excellent point related to preliminary parameters, such as body weight, food and water intake and urine output as general indicators of diabetes. It is best if authors can present those parameters in the result section.

Response:

Food, water intake and urine output for animals were described in the Method section, subheading 2.4

4. Please pay attention to decimal separator. Sometimes author use 'point' as decimal separator (e.g.

BSG 13.5g/kg), while sometime authors use 'comma' (e.g. 0,09 mg/kg; p<0,0001). Please choose one decimal separator and use consistently throughout the text.

Response:
We corrected on the manuscript. Thank you.

Reviewer 3: Methods:
The used dose and the preparation of the extracts are not very clear.

It is not indicated if the extract is concentrated or dehydrated.

The dose of glibenclamide is very low and the one of the extracts she is very high, at least 10 mg/kg of glibenclamide and a maximum of 500 mg/kg for the extracts.

The objective of the work (line 67) mentions that the proinflammatory genes were evaluated, but those are not seen with Elisa

Response:
We added the information regarding how to obtain steeping of black solo garlic, in the Method section, subheading 2.6.
We did mistype in the manuscript regarding proinflammatory gene (line 67). In fact, what we mean is proinflammatory mediators.

Results:

Very limited, very little description of the figures.

Response:

We change the results part.

Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

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Keywords

Black solo garlic, immunomodulator, interleukin, interferon

1. Introduction

Diabetes mellitus is a disease characterized by impaired metabolism of carbohydrates, proteins and fats due to insufficient secretion of insulin. Furthermore, this complication

is caused by decreased insulin sensitivity in the tissues, and it is the leading cause of kidney disease, blindness, and amputations [1]. Diabetes mellitus is one of chronic inflammatory disease that suppresses cellular immunity in diabetic [2].

Diabetes mellitus (DM) is a chronic condition that arises when the pancreas do not create enough insulin, or when the body cannot use the insulin adequately. Insulin deficiency in diabetes increases blood glucose resulting in impaired microcirculation as well as increased oxidative stress resulting in prolonged inflammation [3]. This condition is different from inflammation in general. Therefore, agent that can lower blood glucose as well as an anti-inflammatory is required. Diabetes is associated with high levels of cytokines TNF-α, IL-6, IL-1β, and decreased interferon-γ [4,5]. TNF-α may change permeability of gromerular and albuminaria by promoting local ROS production [6]. IL-6 is a powerful predictor of the development of diabetes complications such as diabetic nephropathy [7].

The body's immunity can increase through nutritious food, adequate rest, and routine exercise. In addition, some plants have beneficial effect as immunomodulator such as garlic (*Allium sativum* L.). Fermentation of garlic produced a black garlic with a sour taste rather than a pungent flavor [8]. Black garlic contains reduced sugars, polyphenols, flavonoids, Amadori and Heyns, leucine, isoleucine, phenylalanine, *S*-allyl-cysteine, and alkaloids content higher than those of fresh garlic [9]. Black garlic have several functions, such as an antioxidants, anti-bacterial, anti-allergic, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [10]. Black garlic increases natural killer cell cytotoxicity and increases the proliferation of B and T lymphocytes and macrophages. The previous study showed that black ordinary garlic extract increases the immune system [11] and recover kidney cells [12].

One of type garlic is single clove or solo garlic (*Allium sativum* 'Solo garlic'), which contains unstable compounds such as allicin [13]. The allicin ratio in a single clove is equivalent to 5-6 of ordinary garlic. In addition, black solo garlic (BSG) contains more *S*-allyl-cysteine six time than fresh garlic, which increase antioxidant activity [14]. Furthermore, the content of polyphenols, vitamins, minerals and flavonoids are increased during the fermentation [8]. However, there has not been any study on immunomodulatory as well as an anti-inflammatory of black solo garlic. Therefore, this study aims to evaluate the immunomodulatory and anti-inflammatory effect of black solo garlic in streptozotocin (STZ)-induced rats. The present study was designed to investigate the changes of proinflammatory mediators (TNF-α, IL-6, IL-1β, IFN-γ) to verify the protective effects of BSG in DM rats.

2. Materials and methods

2.1. Plant

The fresh solo garlics were obtained from the Brebes region in Central Java and validated by the Plant Taxonomy Laboratory at the Faculty of Biology at Jenderal Soedirman University, Indonesia.

2.2 Fermentation of solo garlics

The selected garlics were approximately the same size and were fermented (aged) in the fermentation modified apparatus by using rice cooker. Furthermore, they were arranged in different layers and covered with tissue paper. The rice cooker was set in the warm mode (temperature 60-80^{o}C) left for 21 days and discoloration was observed once every 3 days. After 21 days, the garlic turned into a black chewy texture.

2.3. Preparation of aqueous black solo garlic extract

Black solo garlic were peeled and weighed according to the dose, then mashed using a pestle and mortar. The crushed garlic was placed in a glass, then hot water (at a temperature of 80-90 °C) was added and stirred for 15 minutes. The mixture was filtered to obtain steeping black solo garlic. The steeping were further analysed by using GC-MS, and we found the same results as reported by Tran et al. (2018) [15].

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The study was conducted using a pre- and post-test approach with a control group design after obtaining ethical eligibility from ethics committee, Faculty of Health Sciences, Jenderal Soedirman University No: 152/EC/KEPK/VII/2020. White male Wistar strain rats aged around 6-7 weeks with a body weight range of 150-200 grams were used. They were given *ad libitum* access to standard food and distilled water.

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Streptozotocin was dissolved in 2.5 ml of citrate buffer 0.05 M and the experimental animals were induced by STZ 50 mg/kg body weight on the day 8th. In addition, they were fasted for 6-8 hours before STZ induction. Induction was performed for 3 days.

2.6. Treatments

The total number of animals was 30 rats, which were divided randomly into six group: (i) normal, (ii) STZ-induced rat, (iii) STZ-induced rat treated with glibenclamide 0.09 mg/kg body weight (positive control), STZ-induced rat treated with steeping black solo garlic dose of (iv) 6.5 (low dose), (v) 13.5 (medium dose), and (vi) 26 g/kg body weight (high dose). After STZ-induction for 3 days, the animals were treated with steeping black solo garlic for 7 days on the day 11 to 19$^{th.}$ The same schedule was also applied for glibenclamide treatment (Figure 1). Briefly, the black solo garlic powder was

measured based on the bodyweight of rats to obtain those three doses. The measured powder was diluted in the 3.6 mL hot water and stirred for 15 minutes to obtain steeping of black solo garlic. Steeping black solo garlic was given per oral once at night based on the dose and body weight of each group.

2.7. Measurement of cytokines

Blood was drawn using a hematocrit capillary pipette which was inserted into the *plexus orbitalis* at the corner of the eye in the day 11st and 19th. The ELISA kits were used to check cytokine IL-6, TNF-α, IL-1β, and IFN-γ (BT Laboratories, Shanghai) based on the manufacturer protocol. Briefly, this ELISA kit uses the Sandwich-ELISA principle. The plate has been pre-coated with an antibody specific to rat cytokine. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. The OD value was proportional to the concentration of rat cytokine. Concentration of rat cytokine in the samples was calculated by comparing the OD of the samples to the standard curve.

2.8. Statistical analysis

All data were presented as mean ± SEM. GraphPad Prism 8 (California, US) was used to generate the figure. Furthermore, the mean scores among the treatment groups were compared with the one-way ANOVA with a Tukey post-hoc test. Pre- and post-test treatment were analyzed by t-test. $p<0.05$ was considered as significant.

3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level compared normal group ($p<0.0001$) (Figure 2). The administration of black solo garlic at dose of 6.5 g/kg, 13.5 g/kg and 26 g/kg body weight had significantly reduced the level of IL-1β, IL-6, and TNF-α compared to untreated diabetic rats ($p<0.0001$). Interestingly, the treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to reduce IL-1β, IL-6, and TNF-α level lower than those of glibenclamide. The effects on IL-1β, IL-6, and TNF-α were dose dependent, the reduced level of those cytokines in rats administrated with the medium and high black solo garlic dose (13.5 and 26 g/kg, respectively) being significantly greater than that in the rats receiving low black solo garlic dose (6.5 ~~m~~g/kg). Therefore, treatment of the black solo garlic dose of 13.5 g/kg are equal to those 26 g/kg body weight as an anti-inflammatory agent.

3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction produced a significant decreased of IFN-γ compared to the normal group ($p<0.0001$) (Figure 3). Black solo garlic (13.5 and 26 g/kg)-treated diabetic rats had significantly increased level IFN-γ compared to the untreated diabetic rats ($p<0.0001$). The effects on IFN-γ were dose dependent, IFN-γ levels of rats having been administrated medium or high black solo garlic dose being significantly higher than those in the diabetic rats receiving the low black solo garlic dose. Moreover, the treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to increase IFN-γ level higher than those of glibenclamide.

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [16], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [17]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [18]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they inhibit inflammation by suppressing the activity of the NF-κB signaling [19]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [20]. Flavonoids can function as antioxidants to reduce or terminate free

radical chain reactions [21]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [22]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [23,24].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [25]. These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [26,27]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that have critical role in generating IL-1β is interesting to be explored in the future study.

~~APPENDICES~~

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Desiyani Nani: Conceived and designed the experiments; Analyzed and interpreted the data.

Atikah Proverawati: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sarmoko: Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.~~Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani:~~

~~Methodology, Investigation, Validation, Writing – Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing – Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing – Original Draft, Writing- Reviewing and Editing.~~

Figures

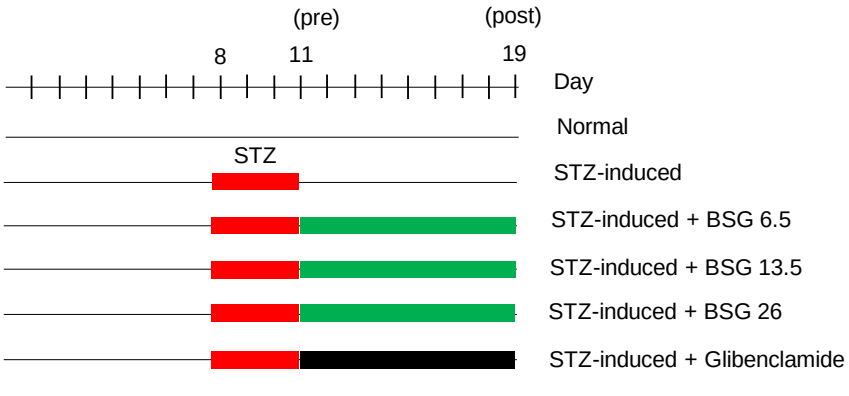

Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo garlic.

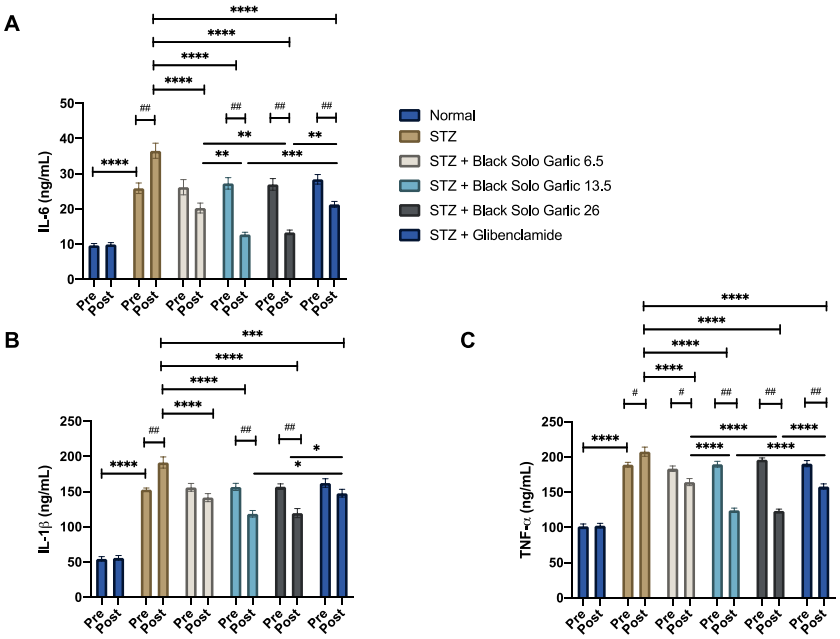

Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6, (B) IL-1β, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26

g/kg doses and glibenclamide 0.09 mg/kg in streptozotocin-induced rats were determined by ELISA. Statistical significance for the difference among groups: **** p<0.0001; *** p<0.0005; *p<0.05. Statistical significance for the difference between the data of pre-test group vs post-test groups: ## p<0.01; # p<0.05

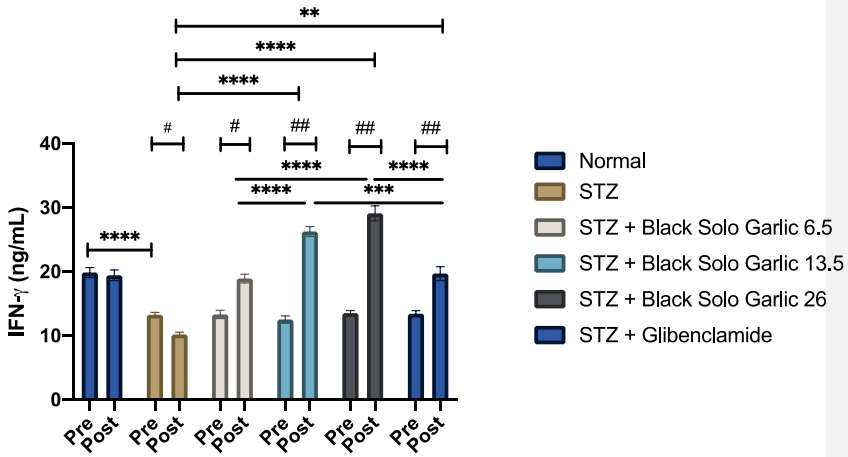

Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0.09 mg/kg in streptozotocin-induced rats was determined by ELISA. Statistical significance for the difference among groups: **** p<0.0001; *** p<0.0005; ** p <0.01. Statistical significance for the difference between the data of pre-test group vs post-test groups: ## p<0.01; # p<0.05

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Immunomodulatory effects of black solo garlic (*Allium sativum* L.) on streptozotocin-induced diabetes in Wistar rats

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Abstract

Diabetes mellitus is a chronic disease that leads to different complications. Therefore, this study aims to investigate the immunomodulatory effects of the black solo garlic on streptozotocin (STZ)-induced diabetic rats. The Wistar rats were grouped into six groups of: normal control, negative control, treatment dose of 6.5 g/kg, 13.5 g/kg, and 26 g/kg body weight, and positive control glibenclamide. In addition to normal control, rats were induced with STZ on day 8 to 11. Also, steeping black solo garlic or glibenclamide was administered on the day 12 to 19. The experimental animals were sacrificed on day 20 and tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interferon gamma (IFN-γ) were measured using ELISA. The results showed that the administration of steeping black solo garlic significantly decreased levels of IL-1β, IL-6 and TNF-α as well as increased IFN-γ with the immunity of STZ-induced rats.

Keywords

Black solo garlic, immunomodulator, interleukin, interferon

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3. Results

3.1. The effect of steeping black solo garlic on pro-inflammatory cytokines

The results showed that STZ induction significantly increased IL-1β, IL-6, and TNF-α level ~~in comparison~~compared ~~to the normal~~ normal group ($p<0.0001$)~~group~~ (Figure 2). The administration of black solo garlic at dose of 6.5 g/kg, 13.5 g/kg and 26 g/kg body weight had significantly reduced ~~50%~~ the level of IL-1β, IL-6, and ~~reduced 37% the level of IL-1β, and~~ TNF-α ~~-~~compared to~~than STZ-induced only group~~untreated diabetic rats ($p<0.0001$). Interestingly, t~~T~~he treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to reduce IL-1β, IL-6, and TNF-α ~~-~~level lower ~~more significant~~ than those of glibenclamide ~~(Figure 2B)~~. The effects on IL-1β, IL-6, and TNF-α were dose dependent, the reduced level of those cytokines in rats administrated with the medium and high black solo garlic dose (13.5 and 26 g/kg, respectively) being significantly greater than that in the rats receiving low black solo garlic dose (6.5 mg/kg). ~~Moreover, the treatment of black solo garlic was able to reduce TNF-α levels~~

~~in all doses (Figure 2C).~~ Therefore, treatment of the black solo garlic dose of 13.5 g/kg ~~body weight~~ are equal to those 26 g/kg body weight as an anti-inflammatory agent.

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3.2. The effect of steeping black solo garlic on IFN-γ

Streptozotocin induction produced a significant decreased of IFN-γ compared to the normal group ($p<0.0001$) (Figure 3). Black solo garlic (13.5 and 26 g/kg)-treated diabetic rats had significantly increased level IFN-γ compared to the untreated diabetic rats ($p<0.0001$). The effects on IFN-γ were dose dependent, IFN-γ levels of rats having been administrated medium or high black solo garlic dose being significantly higher than those in the diabetic rats receiving the low black solo garlic dose. Moreover, the treatment of black solo garlic at dose of 13.5 g/kg and 26 g/kg body weight was able to increase IFN-γ level higher than those of glibenclamide.

~~Streptozotocin induction reduced IFN-γ level in the rats. In contrast, black solo garlic in all doses increased IFN-γ level (Figure 3). Black solo garlic at a 13.5 and 26 g/kg body weight doses showed the significant increased of IFN-γ levels than those of STZ-induced only group.~~

4. Discussion

The administration of steeping black solo garlic decreases the level of IL-1β, IL-6 and TNF-α and increases IFN-γ in experimental animals. These results suggested that black solo garlic is potential to be used as an immunomodulator and antiinflammation in diabetic condition to prevent complication.

Induction of STZ generates free radicals which oxidize β-pancreatic cells by means of alkylating DNA, damaging mitochondria, and inhibiting the O-GlcNAcase enzyme. The oxidation process induces toxicity through a free radical chain reaction. This results in an inflammatory process to organ malfunction [16], which causes the release of major pro-inflammatory cytokines such as IL-1β and TNF-α. The present study showed that the administration of steeping black solo garlic significantly reduced levels of IL-1β, IL-6 and TNF-α and increased IFN-γ in experimental animals.

Black solo garlic contains *S*-allyl-cysteine that has pharmacological effect as an antidiabetic, antioxidant, and anti-inflammatory with a higher bioactivity than regular types. Furthermore, the *S*-allyl-cysteine level with distilled water is higher than ethanol solvent [17]. Black solo garlic contains organosulphur compounds with potent antioxidant activity and free radical scavengers [18]. During inflammation, many pro-inflammatory mediators are generated and the administration of black solo garlic suppresses them to avoid tissue damage. Antioxidant compounds such as *S*-allyl cysteine, *S*-allyl mercaptocysteine, and allicin exhibit antioxidant activity and they

inhibit inflammation by suppressing the activity of the NF-κB signaling [19]. Meanwhile, *S*-allyl-cysteine has been proven to scavenged superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuron, as well as hypochloric acid and singlet oxygen in microglial cells.

Chemical compounds content of solo garlic is similar to ordinary type; however, some compounds have higher amounts such as flavonoids, total phenols, *S*-allyl-cysteine, and minerals [20]. Flavonoids can function as antioxidants to reduce or terminate free radical chain reactions [21]. *S*-allyl cysteine ameliorates cells by regulating peroxisomal proliferator activator receptor-α (PPAR-α), sterol regulatory element binding protein 1c (SREBP-1c), and decreasing levels of reactive oxygen species [22]. In addition, black solo garlic contains minerals such as Cu, Mn, and Zn, which have important role in the activity of oxidant enzymes such as superoxide dismutase. The antioxidant activity reduces reactive oxygen species and increases glutathione peroxidase, catalase, superoxide dismutase, reduced glutathione (GSH) and malondialdehyde [23,24].

Black garlic suppresses the toll-like receptor 4 (TLR4) signals in macrophages. TLR4 acts to activate myeloid differentiation factor 88 (MyD88). It releases pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [25]. These various mechanisms underlie several studies which explaining the role of black garlic in suppressing the formation of TNF-α, IL-1β, and interferon-γ [26,27]. Although BSG affected TLR4, the effect on NLRP as a component of inflammasome that have critical role~~lp~~ in~~to~~ generating~~e~~ IL-1β is interesting to be explored in the future study.

APPENDICES

Acknowledgements

The authors are grateful to ~~the~~ Dr. Arif Fadlan for his comment on this manuscript. This research was funded by LPPM Jenderal Soedirman University through Institution Research Grant (RISIN) No. T/324/UN23.18/PT.01.03/2020.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Saryono: Conceptualization, Methodology, Data curation, Resources, Supervision, Writing- Reviewing and Editing. Desiyani Nani: Methodology, Investigation, Validation, Writing - Original Draft. Atikah Proverawati: Methodology, Investigation, Project Administration, Writing - Original Draft. Sarmoko: Resources, Visualization, Formal Analysis, Writing - Original Draft, Writing- Reviewing and Editing.

Figures

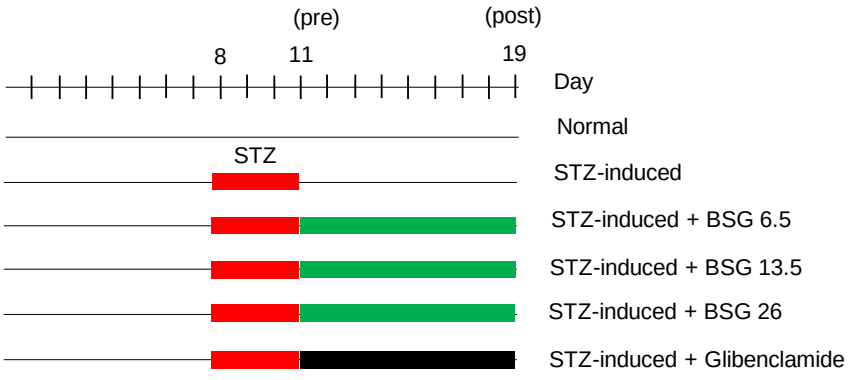
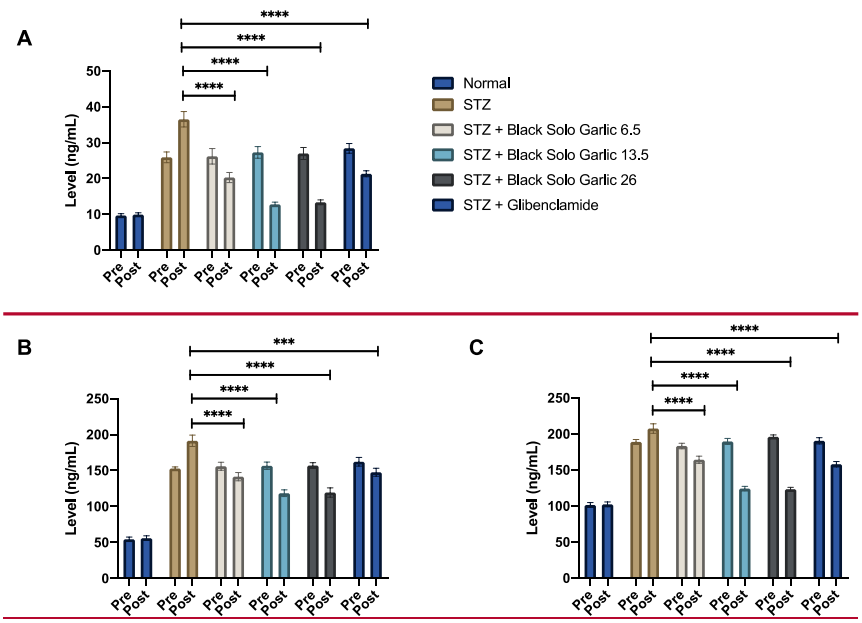


Figure 1. Study design in animal experiments. STZ, streptozotocin; BSG, black solo garlic.



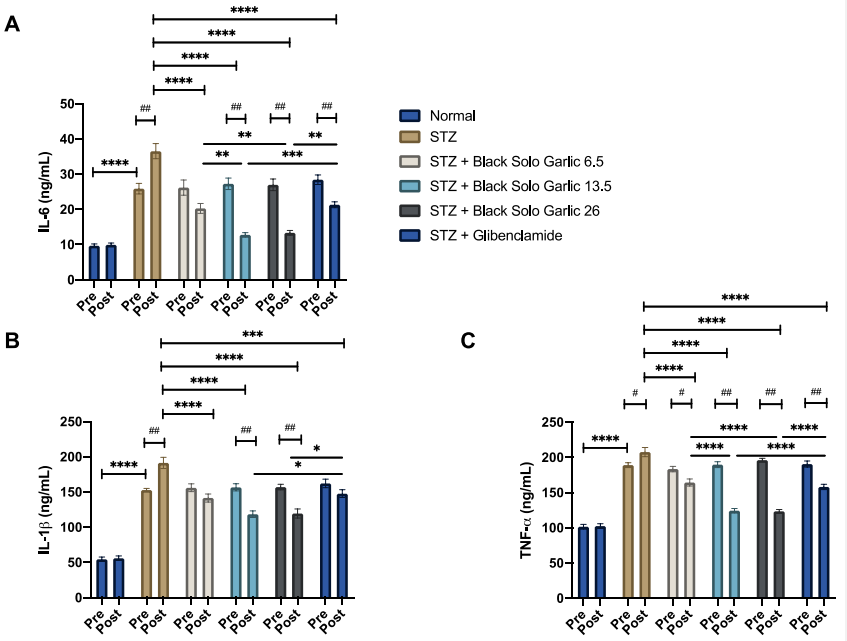
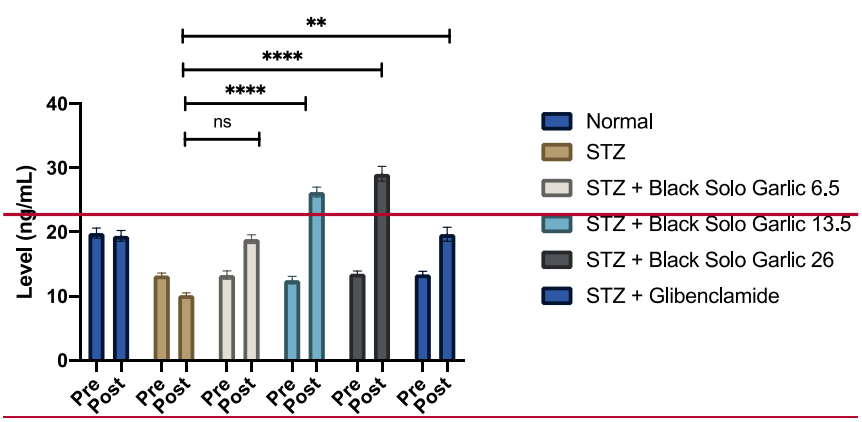


Figure 2. Treatment of black solo garlic reduced pro-inflammatory cytokines. (A) IL-6, (B) IL-1β, (C) TNF-α level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,09 mg/kg in streptozotocin-induced rats were determined by ELISA. Statistical significance for the difference among groups: **** p<0.0001; *** p<0.0005; *p<0.05. Statistical significance for the difference between the data of pre-test group vs post-test groups: ## p<0.01; # p<0.05

~~**** p <0,0001; *** p<0,0005~~



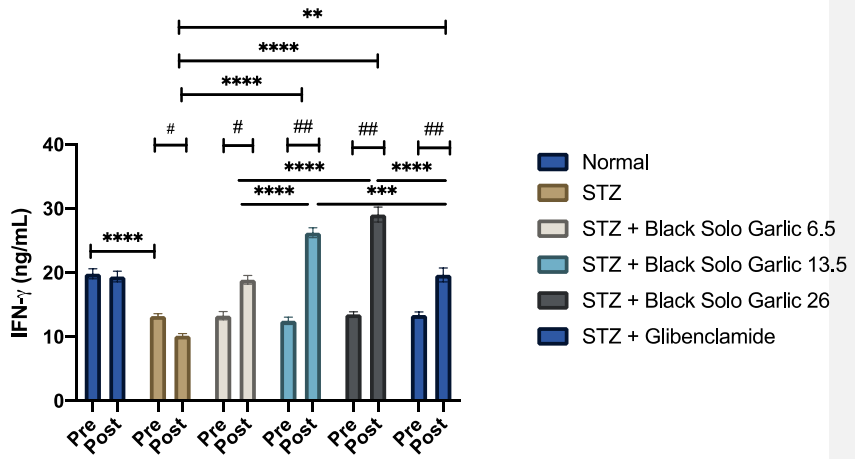


Figure 3. Treatment of black solo garlic increased IFN-γ. IFN-γ level in the treatment of black solo garlic in 6.5, 13.5, 26 g/kg doses and glibenclamide 0,~~,~~09 mg/kg in streptozotocin-induced rats was determined by ELISA. Statistical significance for the difference among groups: **** p<0.0001; *** p<0.0005; ** p <0.01. Statistical significance for the difference between the data of pre-test group vs post-test groups: ## p<0.01; # p<0.05

~~**** p<0,0001; ** p <0,01; ns = not significant.~~

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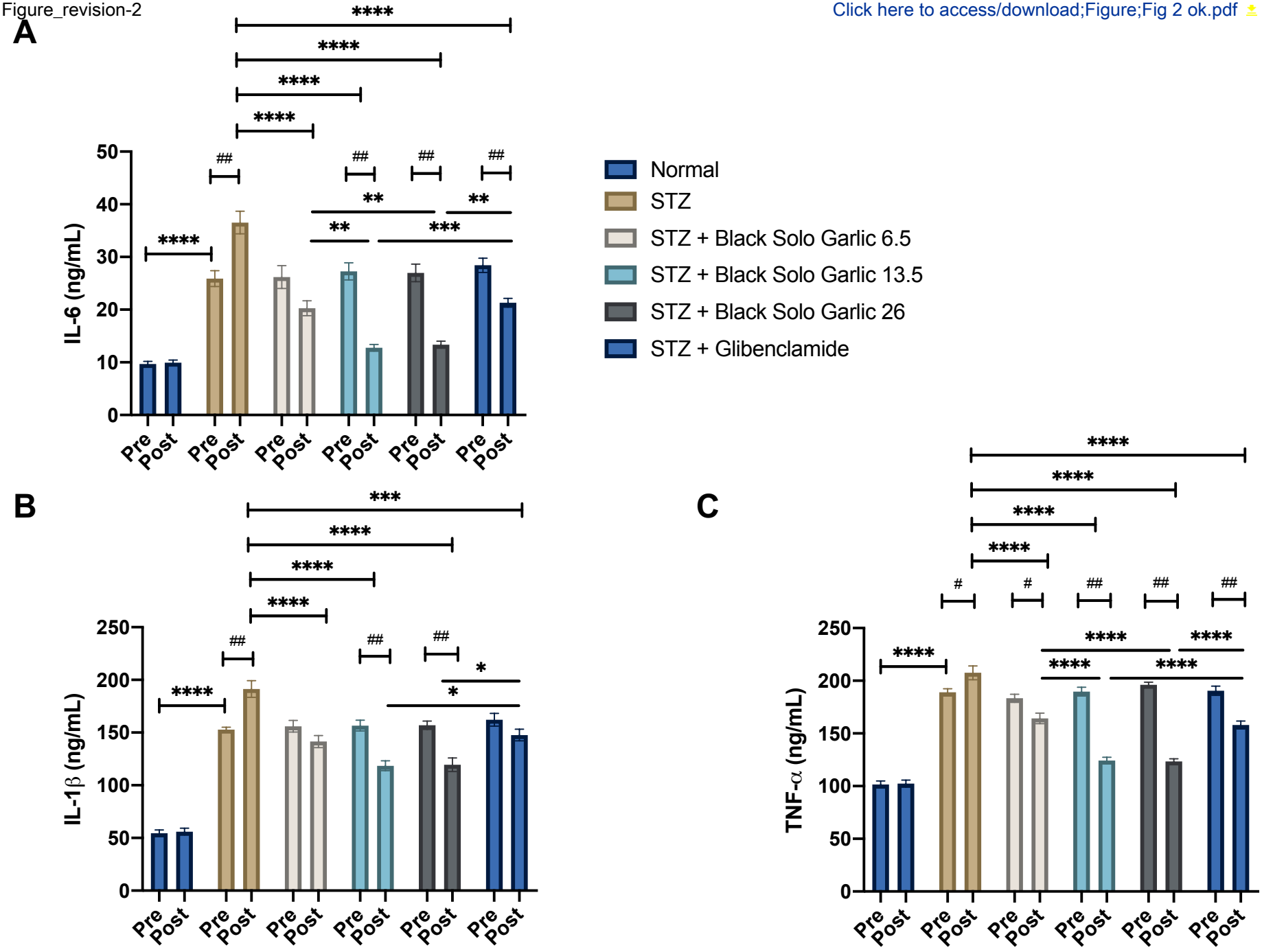

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