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The effect of *Tithonia diversifolia* extract against the level of nitric oxide in streptozotocin-nicotinamide-induced rats model

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ABSTRACT

Background: One indication of microvascular dysfunction in diabetes mellitus (DM) is decreased nitric oxide (NO) levels. Tithonia diversifolia leaves (TDL) extract has been scientifically demonstrated to decrease glucose levels in diabetic rats.
Objective: This study aims to examine the effect of TDL extract on NO levels in diabetic rats.
Methods: True experimental with pre-post test control group design used 25 Sprague-Dawley rats. Its were divided into 5 groups: healthy control (K.1); diabetic control (K.2); diabetes rats with ethanol extract of TDL at a dose of 25 (K.3); 50 (K.4); and 100 mg/kg BW (K.5) for 28 days. We use a combination of nicotinamide (230 mg/kg BW) and streptozotocin (65 mg/kg BW) to induce diabetes mellitus. The blood serum was taken before and after extract administration. NO level was assessed using spectrophotometry. Paired T-test, Wilcoxon, one-way ANOVA, and post hoc LSD were performed for statistical analysis.
Results: There were significant differences in NO levels before and after treatment in all groups unless K.5 (100 mg/kg BW). Furthermore, there was no significant difference in NO levels in the healthy control and the K.5 group but significant differences in other groups.
Conclusion: TDL extract can prevent the decrease in NO level in diabetic rats model with the effective dose of 100 mg/kg BW.

Latar Belakang: *Salah satu tanda disfungsi mikrovaskular pada diabetes melitus (DM) adalah penurunan kadar nitrogen monoksida (NO). Ekstrak daun Tithonia diversifolia (TDL) telah terbukti secara ilmiah dapat menurunkan kadar glukosa darah pada DM*
Tujuan: *Penelitian ini bertujuan untuk mengetahui pengaruh ekstrak TDL terhadap kadar NO pada tikus diabetes.*
Metode: *True experimental dengan pre-post test control group menggunakan 25 ekor tikus Sprague-Dawley. Mereka dibagi menjadi 5 kelompok: kontrol sehat (K.1); kontrol diabetes (K.2); tikus diabetes dan diberi ekstrak etanol TDL dengan dosis 25 (K.3); 50 (K.4); dan 100 mg/kgBB (K.5) selama 28 hari. Kami menggunakan kombinasi nikotinamida (230 mg/kgBB) dan streptozotocin (65 mg/kgBB) untuk*

menginduksi DM. Serum darah tikus diambil sebelum dan sesudah pemberian ekstrak. Kadar NO diukur menggunakan spektrofotometri. Data dianalisis menggunakan uji paired T-test, Wilcoxon, one-way ANOVA, dan post hoc LSD

Hasil: *Terdapat perbedaan signifikan pada kadar NO sebelum dan sesudah perlakuan di semua kelompok, kecuali kelompok K.5 (100 mg/kg BB). Lebih lanjut, tidak terdapat perbedaan signifikan kadar NO pada kelompok kontrol sehat dengan kelompok K.5, tetapi untuk kelompok lainnya terdapat perbedaan signifikan.*

Kesimpulan: *Ekstrak TDL dapat mencegah penurunan kadar NO pada tikus model diabetes dengan dosis efektif 100 mg/kgBB.*

INTRODUCTION

Diabetes mellitus (DM) is caused by an inability of the insulin function or pancreas to produce insulin. Worldwide, around 1.6 million death was caused by DM in 2016.[1] The incidence increased from 6.9% (2013) to 8.5% (2018) among those aged >15 years in Indonesia.[2] The prevalence of type 2 diabetes mellitus (T2DM) takes place in around 90% of the total DM patients.[3]

Vascular trauma or stress led to endothelial cells producing nitric oxide (NO) as a vasodilator modulated by endothelial nitric oxide synthase (e-NOS). In DM, several mechanisms influence microvascular dysfunction, including decreasing NO levels and escalating reactive oxygen species (ROS).[4] Several studies demonstrated that the decreased NO levels were caused by the reduction of e-NOS to produce NO and the escalation of NO degradation by ROS activity.[5]

Tithonia diversifolia (Hemsl.) leave (TDL) is known by Indonesian as "kembang bulan", which is often used as an anti-diabetic plant. The anti-diabetic properties compounds in TDL extract are sesquiterpenoids. This active substance can reduce insulin resistance.[6] The previous study suggested that other high-level components in TDL extract were tannins, flavonoids, and phenols. Therefore, this plant can be used as a preventive therapy for various diseases such as DM and cardiovascular disease.[7]

Flavonoids can be used for DM therapy and its complications because flavonoids have high antioxidant properties. In vascular, especially NO, flavonoids protect and prevent the formation of uncoupling NOS so it can maintain the level of NO.[8] In addition to oxidative stress, flavonoids can protect NO from its inactivation due to their action with superoxide, preventing NO degradation by ROS activity.[9]

Tithonia diversifolia is an endemic plant in tropical countries such as Indonesia. Therefore, this plant is accessible as raw material for anti-diabetic drug development based on local wisdom. However, no studies have investigated the effect of BP on NO levels as an indicator of microvascular dysfunction. This study is expected to examine the effect of BP on microvascular events by measuring NO levels in blood serum.

METHODS

This study used a pre and post-test with a control group design. Our data collection used a completely randomised. Twenty-five male Sprague Dawley rats were divided into 5 groups with 5 rats each: healthy control (K.1); diabetic control (K.2); treatment group with TDL extract in stratified doses administration of 25 mg/kg body weight (BW) (K.3); 50 mg/kg BW (K.4); and 100 mg/kg BW (K.5). DM was induced in the diabetes control group and TDL extract using intraperitoneal injection of nicotinamide (NA) at a dose of 230 mg/kg BW and followed by streptozotocin (STZ) at a dose of 65 mg/kg BW 20 minutes later. The entire process in this study has met the ethical rules according to the permission of the ethics committee of the Faculty of Medicine, Universitas Jenderal Soedirman (Number: 144/KEPKNIU2020).

Extract production

One kilogram of dry powdered TDL was macerated with 2 litres of 70% ethanol for 72 hours. The filtrate was separated using a filtration (Buchner funnel), and maceration was repeated 3 times. The second and third macerations were carried out for 24 hours. The filtrate is combined and evaporated using a rotatory evaporator. The extract was made at the Pharmacology Laboratory Health

Sciences Faculty, and plant determination tests were carried out at the Biology Laboratory of Universitas Jenderal Soedirman.

Animal preparation, acclimatisation, and treatment

Experimental animals were randomly selected based on inclusion criteria (male rats, age 8-10 weeks, body weight ≥150 grams, blood glucose ≤150 mg/dl before induction) and exclusion (blood glucose >150 mg/dl before induction and body weight <150 grams). Then those animals were grouped into 5 groups. They were acclimatised for a week for environmental adaptation at the animal house of the Medical Faculty. Animals were placed in cages at room air temperature and had a light-dark cycle of 12 hours. The animals were fed and drank on an ad libitum.

On day 8, animals were weighed to assess whether they met the inclusion criteria. Blood sampling was conducted to assess rats' glucose and NO levels before treatment. Then, diabetes induction was carried out. TDL extract was administered on day 8 for 28 days according to a stratified dose.

Sampling, animal sacrifice, and NO serum assessment

On day 35, 3 cc of blood rats were taken and the rats were sacrificed. Blood was centrifuged to obtain serum. UV-Vis spectrophotometry at 550 nm wavelength and a NO colourimetric assay kit by Elabscience were used to assess NO levels from serum.

Data analysis

The data were analysed using paired T-test, Wilcoxon, one-way ANOVA, and post hoc LSD with a level 95% confidence interval (95%CI).

RESULTS

The NO assessment results were carried out 2 times, before (pre-test) and after (post-test) administered TDL extract. Figure 1 demonstrated significant differences in NO levels before and after treatment in groups K.1, K.2, K.3, and K.4 ($p<0.05$). While in group K.5, there is no significant difference in NO levels before and after treatment ($p>0.05$).

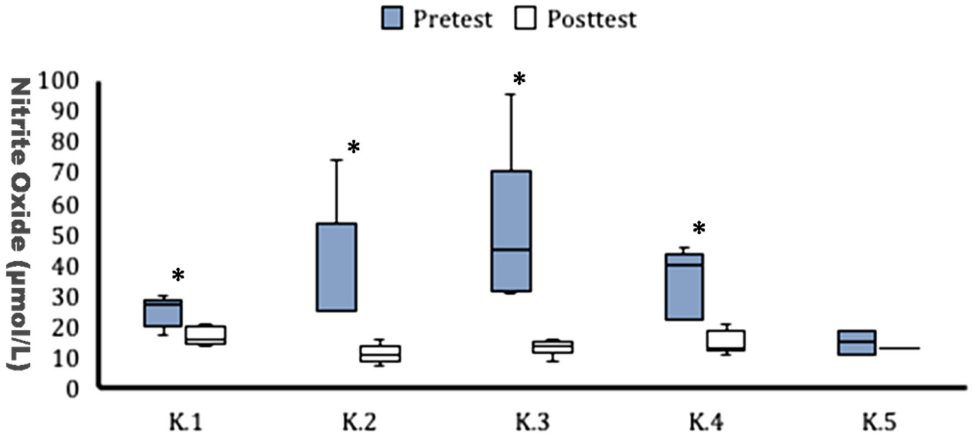

Figure 1. Levels of NO (µmol/L) before and after Tithonia divorsifolia leaves extract administration (n = 25).
Note: K1 = Healthy control; K2= diabetic control; K3= treatment group with dose administration of 25 mg/kg BW; K4= treatment group with dose administration of 50 mg/kg BW; K5 = treatment group with dose administration of 100 mg/kg BW
*(Significant difference between before and after treatment p<0,05 with p-value 0,048, 0,042, 0,001, 0,07 for K1, K2, K3, and K4, respectively)

Table 1. The effect of Tithonia diversifolia leaves extract on the reduction of NO (µmol / L) levels in Sprague Dawley rats (n = 25)

Group	Reduction of NO level (mean±SD µmol/L)
Healthy control	8,00±6,36*
Diabetic control	25,00±19,21+
K.3	36,40±26,88*x
K.4	19,60±8,50
K.5	7,60±6,88+x

Notes:
*Significant difference between groups 1 and 3 p=0.008 (CI -0,813 – -0,144),
+Significant difference between groups 2 and 5 p=0,040 (CI 0,018 – 0,688),
xSignificant difference Between Groups 3 And 5 p=0,004 (CI 0,182 – 0,851)

Table 1 demonstrates the mean value reduction of NO levels obtained from the difference before and after treatment (delta NO). There was a significant difference in the reduction of NO levels between groups. The reduction of NO levels in group 5 was significantly lower than in groups K.2 and K.3 ($p<0.05$). Meanwhile, there was no significant difference in the reduction of NO levels in group K.5 compared to group 1 ($p>0.05$).

DISCUSSION

This study's results demonstrated a significant effect of TDL extract on NO levels in diabetic rats compared to before administering the extract. This study is in line with a previous study that the supplementation of flavonoids orally for 3-4 weeks can suppress oxidative stress that occurs in the vascular system, thereby reducing levels of ROS. This condition prevents NO degradation.[10]

Tithonia diversifolia was demonstrated to reduce the blood sugar levels of diabetic rats.[11,12] In a previous study, TDL extract at doses of 25 mg/kg BW, 50 mg/kg BW, and 100 mg/kg BW significantly reduce blood sugar levels.12 However, in this study, with a similar treatment, TDL extract administration at a 25 mg/kg BW dose was insufficient to increase the NO level, as shown in Table 1. Meanwhile, K.4 and K.5 groups demonstrated an increase in NO levels compared to the diabetic control group. Furthermore, the increase in NO level in the K.5 group was better than in K.1. Thus, administration of TDL extract at a dose of 50 mg/kg BW and 100 mg/kg BW to STZ-NA-induced rats could prevent a decrease in NO level, with a significant result occurring in the K.5 group, the dose of 100 mg/kg BW. This finding is in line with other studies, which demonstrated that diabetic rats administered TDL extract at doses of 50 mg/kg and 100 mg/kg BW had a significant ability to lower blood glucose compared to a dose of 25 mg/KgBB.[12]

This study demonstrated that TDL extract had a positive effect on NO levels. Several studies suggested this positive correlation because TDL contains high flavonoids, alkaloids, phenolic compounds, tannins, and terpenoids. These components can neutralise the instability of the ROS structure by donating an electron, thus preventing ROS from reacting with NO-induced peroxynitrite formation.[10]

TDL extract also increased NO levels by increasing e-NOS expression and suppressing its inhibitors. Flavonoids act as acetylcholine esterase inhibitors and play a role in e-NOS down-regulation.[13,14] The same study also demonstrated that quercetin, which is included in the flavonoid class, can directly increase the expression of e-NOS, thereby increasing NO production.[14] In addition, alkaloids play a role in increasing e-NOS expression by inhibiting L-NG-nitro-L-arginine (L-NNA), which is an

e-NOS inhibitor.[15]

The increase in NO levels also occurs because flavonoids and phenolics protect tetrahydrobiopterin (BH4) levels. BH4 acts as a NOS cofactor in the NO synthesisprocess. Flavonoids and phenolic compounds could protect BH4 from free radicals, such as peroxynitrite, that cause BH4 oxidation. Flavonoids and phenolic compounds separate the peroxynitrite formation pathway, preventing BH4 oxidation.[16] Flavonoids also play a role in asymmetric dimethylarginine (ADMA) catabolism which functions as a competitive NOS inhibitor.[17] The ADMA activity decreased due to an increase in the activity of antioxidant enzymes, such as superoxide dismutase (SOD).[18] Several studies have shown that TDL extract increases SOD activity due to increased activity of peroxisome proliferator-activated receptors γ (PPAR γ).[19,20,21]

A previous study demonstrated that TDL could act as PPAR γ dual agonists because they contain tirotundin and tagitinin A. They are directly binding to the PPAR γ ligand-binding pocket. This bond could be formed due to hydrophobic contact and significant hydrogen bonding interactions with its hydrogen transfer.[19] Several studies also evidenced that PPAR γ activation could increase NO production through insulin receptors activation, thus activating the phosphatidyl inositol-3 kinase/Akt/eNOS pathway or increasing 90-eNOS protein interactions, phosphorylation of eNOS Ser1177 and Thr495 in endothelial cells in increasing eNOS expression.[21,22] Another study demonstrated that PPAR γ regulates NO production through the NOS pathway, indicating NO levels increased due to increased PPAR γ activation.[23]

K.3 group has a significant difference from K.1 group and not significant in K.2 group. It was found that the dose of 25 mg/kg BW had a much greater reduction than the diabetic control group. These conditions suggested that, at a dose ≥50 mg/kg BW, it could increase the expression of e-NOS because the concentration and dose of higher antioxidant content, such as quercetin 10 μM, could increase the phosphorylation of Akt-eNOS.[24] In addition, doses lower than 50 mg/kg BW demonstrated that the suppression effect of inducible nitric oxide synthase (i-NOS) produced by macrophages was more dominant than the e-NOS expression rate. Macrophages could produce superoxide, thereby increasing the interaction of NO and superoxide to form peroxynitrite, reducing NO levels.[25] According to a previous study, aqueous TDL extract administration at 10 mg/kg BW caused a change in haematological parameters, such as a decrease in leukocytes and neutrophils. This finding indicated that the lower dose of TDL can inhibit neutrophil migration to the inflammation site, thus reducing macrophage activity.[26] In addition, TDL extract at 5 μg/mL dose could reduce the expression of TGF-β, resulting in macrophage activation reduction and preventing NO production by i-NOS.[27] Furthermore, taginin C content in TD could reduce VEGF expression.[28] A previous study reported that TDL extract could reduce VEGF levels at 5 μg/mL, 10 μg/mL, and 20 μg/mL.27 Similar to NO, increased VEGF expression also plays a role in the progress of microvascular dysfunction.[29]

Different results were in the K.5 group, which had a significantly higher increase in NO than the diabetes control group. The TDL extract administration at a dose of 100 mg/kg BW effectively increases the NO level, even though the result is close to the NO level in the healthy control group. Previous studies have demonstrated that the higher the extract dose, the greater the effect. According to a previous study, the concentration of the active compound can react at higher doses, so its suppressive and anti-inflammatory properties are better than lower doses.[30] Based on this study, the most effective and safe dose of TDL extract was 100 mg/kg BW. However, the ability to reduce blood sugar levels at this dose is still much lower than the administration of metformin at a dose of 63 mg/kg BW.[31]

DM causes microvascular dysfunction, which is characterised by decreased NO level. Administration of TDL extract at a dose of 100 mg/kg BW can inhibit the reduction of NO levels in diabetic rats, thereby preventing microvascular damage. However, further study is needed, such as assessing other markers such as endothelin-1 and VEGF to investigate the preventive effect of TDL extract.

CONCLUSION

This study showed a significant effect of TDL extract administration on NO levels reduction in diabetic rats with an effective dose of 100mg/kg BW.

CONFLICT OF INTEREST

We have no conflicts of interest to disclose

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