

Research Article

Asymetric Dimethylarginine Levels and Dimethylarginine Dimethylaminohidrolase 2 Expression on Nitric Oxide Synthesis Pathway in Sprague Dawley Rats Induced Nicotinamide and Streptozotocin by Giving *Physalis angulata* L

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Abstract: Hyperglycemia in patients with diabetes will increase oxidative stress to the endothelium and cause endothelial dysfunction. Endothelial dysfunction related to oxidative stress due to hyperglycemia can be improved through several mechanisms, including the synthesis of nitric oxide pathway (NO). *Physalis angulata* L is one kind of plant that has been used as an antidiabetic, due to fisalin. The Objective of this study is to determine the levels of Asymetric Dimethylarginine (ADMA) and expression Dimethylaminohidrolase 2 (DDAH2) on Nitric Oxide (NO) synthesis pathway in Sprague Dawley rats induced Nicotinamide (NIC) - Streptozotocin (STZ) by giving *P. angulata* L standardized fisalin. The study was true experimental research design with a post-test only control group design. Subjects were 30 male Sprague-Dawley rats induced STZ (45 mg/BW) and NIC (110 mg/BW). Giving *P. angulata* L standardized fisalin with a dose of 20 mg/BW was conducted for 21 days. Insulin injections of 200 mg/BW and verapamil injections of 1 mg/BW. It is found that there was a significant result ($p = 0.009$; $r = 0.596$ and $R^2 = 0.355$) and the effect of variation of 35.5% of the group ADMA levels. There was a significant result ($p = 0.004$; $r = 0.505$ and $R^2 = 0.255$) and the effect of variation was 25.5% of the group DDAH2 expression in circular position and there was no effect ($p = 0.284$; $R = 0.202$ and $R^2 = 0.041$) on the expression of group variation DDAH2 longitudinal position. In conclusion, giving of *P. angulata* L standardized fisalin can improve NO synthesis through a decrease in ADMA levels and increased expression of DDAH2.

Keywords: ADMA, DDAH2, NO, Fisalin, *Physalis angulata* L, Verapamil, Insulin

INTRODUCTION

Economic impact of diabetes seen in medical expenses and loss of income, and due to complications such as blindness and vascular disease[1]. Hyperglycemic condition caused by diabetes leads to complications of some diseases caused by endothelial dysfunction through various metabolic pathways such as polyols, heksosamin, advanced glycosylation end-products (AGEs), protein kinase C (PKC) and asymmetric dimethylarginine (ADMA). Mechanism involving ADMA associated with increased expression of Dimethylarginine Dimethylaminohidrolase (DDAH)[2-4]. Dimethylarginine Dimethylaminohidrolase 2 very sensitive to oxidative stress. Oxidative stress on the state of insulin resistance, obesity, hypertension and hyperglycemia can reduce DDAH 2 activity and

increased levels of ADMA. Asymmetric dimethylarginine is an endogenous inhibitor of the NOS, so as a result of decreased activity of DDAH and ADMA accumulation will result in a decrease in the expression of eNOS that would interfere with the production of NO [4-6].

Nitric oxide plays a role in calcium homeostasis through several mechanisms: inducing the entry of calcium into cells, increasing regulation of cyclic nucleotides on Ca channels and activates cGMP resulting influx of calcium ions, calcium signals control the process of proliferation and induction of mediator in the process of arachidonic acid (AA). Increased levels of calcium in the cell is known to stimulate an increase in eNOS expression, so it takes some organic

compounds, calcium channel cover, one of which is verapamil [7].

Various natural plant Indonesia is very rich in antioxidants and can be utilized for the management of DM. Among these plants is *Momordica charantina*[8], *Phyllanthus niruri*[9], *Allium sativum*[10] and *Physalis angulata L* [11-13]. Empirically *P. angulata L* can be used for anti-inflammatory, antimicrobial, anticancer, antihypertensive and antidiabetic [14].

Research *P. angulata L* standardized as an antioxidant related fisalin ADMA, DDAH2 on NO synthesis pathway has never been found. The study on the expression levels of ADMA and DDAH2 on NO synthesis pathway by administration of *P. angulata L* standardized fisalin with insulin and or verapamil should be induced in experimental animals Nicotinamide - Streptozotocin (STZ).

METHODOLOGY

Sprague Dawley strain male rats aged 12 weeks, weighing 180-200 g maintained stable individually, cleaned every day. Maintenance room ventilated area, 28-32°C room temperature, relative humidity 98%, with the arrangement of dark and light 12 hours. AIN-93 feeding and drink water ad libitum. Adapted animals in cages one week prior to treatment.

Sprague Dawley strain male rats tail number 30, made diabetes mellitus type 2 with nicotinamide + STZ induction. Induction of nicotinamide 110 mg / BW given 8 days to fifteen minutes (15 ') and then given an injection of STZ at a dose of 45 mg / kg BW. After three days of blood sugar measurements to make sure the rats are already experiencing hyperglycemia. A number of 30 hyperglycemic rats were divided into six groups randomly. Each group consisted of 5 rats. Treatment *P. angulata L* standardized fisalin at a dose of 20 mg/BW/day for 21 days. On day 30, was given an

injection of insulin 200 mg/BW and verapamil injections of 1 mg / kg BW. On day 10th, the 31st and 32nd levels of ADMA examination, examination DDAH2 expression on day 32. Normality test data by kolmogorov smirnof while knowing the difference by ANOVA and regression analysis.

RESULTS AND DISCUSSION

The results showed that the experimental animals had traits that characterized by hyperglycemia polydipsi, polydipsi and polyuria. Some of the variables that affect the parameters controlled study in a manner that ensures herbs are used, the process of making extract and kuantifikasinya, knowing animal weight, feed intake, the amount of urine and blood sugar levels.

Giving group variation does not give effect to the animal body weight, feed intake, urine output but an impact on blood sugar levels. The results of the regression test showed the results of p = 0.013 with a positive direction and strength being (r = 0.525) and the value of R2 = 0.276. Therefore, the provision of variation of 27.6% group gives effect to decrease blood sugar levels and 72.4% influenced by other factors.

In hyperglycemic conditions there will be a mechanism for the increase in blood sugar levels that are not only derived from the digestion and absorption of food, but also derived from gluconeogenesis and glycogenolysis. When needs are not fulfilled then the compound carbohydrate non carbohydrate through gluconeogenesis pathway to produce glucose. Increased gluconeogenesis is probably caused by a state of lipolysis. Lipolysis is dismantling fats into fatty acids to be used by the network without the need for insulin. Excess fatty acids are converted into triglycerides in the liver resulting in the increase in triglyceride levels. Increased blood sugar levels will also increase the formation of superoxide free radicals [15].

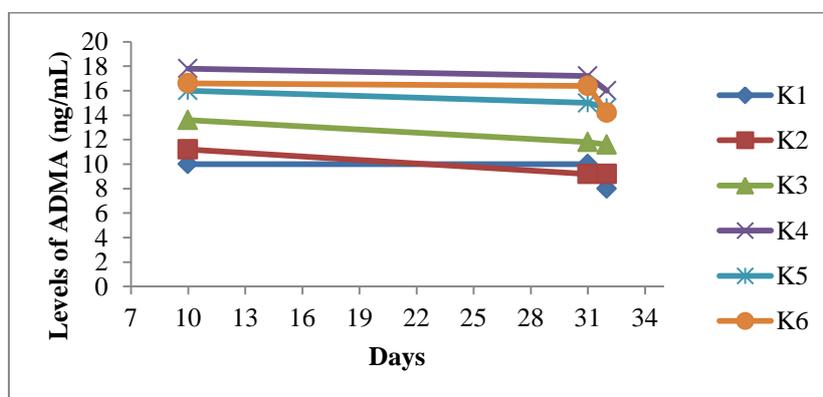


Fig-1: Levels of ADMA day 10, day 31 and day 32.

Note:

K1 = distilled water, K2 = distilled water + insulin, K3 = distilled water + verapamil
 K4 = *P. angulata L* standardized fisalin, K5 = *P. angulata L* standardized fisalin + insulin, K6 = *P. angulata L* standardized fisalin + verapamil

The effect of *P. angulata* L standardized fisalin against the levels of ADMA. Examination conducted ADMA levels as a marker of oxidative stress, which would decrease the activity DDAH2 and resulted in the accumulation of ADMA. Increased levels of ADMA to inhibit eNOS activity resulting in decreased levels of NO. Measurement of ADMA levels performed on day 10, 31 and 32 by ELISA. Complete results can be seen in Figure 1.

Figure 1 shows the average levels of ADMA day 10th, 31st and 32nd. Average levels of ADMA day 10, day 31 and day 32 showed a decrease (14.20 + 4.44 ng / mL be 13.27 ± 4.67 ng/ml and 12.27 ± 4 , 40 ng/mL). Decreased levels of ADMA in all groups.

Results of analysis of variance (ANOVA) both between and within treatments showed significant results on day 10,31 and 32 (p = 0.014, p = 0.009 and p = 0.009) in succession. There was a difference between treatment groups with ADMA levels on day 10: K1-K4 (p = 0.003), K1-K5 (p = 0.017), K1-K6 (p = 0.009), K2-K4 (p = 0.009), K2-K6 (p = 0.029). There was a difference between treatment groups with ADMA levels on day 31: K1-K4 (p = 0.006), K1-K5 (p = 0.048), K1-K6 (p = 0.013), K2-K4 (p = 0.003), K2-K5 (p = 0.024), K2-K6 (p = 0.006), K3-K4 (p = 0.034). There was a difference between treatment groups with ADMA levels on day 32: K1-K4 (p = 0.002), K1-K5 (p = 0.007), K1-K6 (p = 0.011), K2-K4 (p = 0.006), K2-K5 (p = 0.025), K2-K6 (p = 0.037). Regression analysis showed a significant result (p = 0.009; r = 0.596 and R² = 0.355). This means that there were an influence of variation of 35.5% of the group ADMA levels, indicated by the value of r is.

Based on the results, that there were a trend to decreased levels of ADMA over time *P. angulata* L. The difference decreased levels of ADMA day 10, day 31 and day 32, K2 is better than K5 ($\Delta 2$ ng/mL > $\Delta 1$, 4 ng/mL). Decrease in ADMA levels after administration of insulin on the K5 better than the K2. There was a significant difference in the K2-K5 showed with p = 0.024, p = 0.025 at day-31 and day-32 consecutive (Appendix 18). Average expression DDAH2 the circular and longitudinal position better than K2 K5 (19.69% + 7.03% > 16.30% + 0.97%; 16.38% + 2.86% > 14.92% + 11. 61%) consecutive on day 32.

Based on the results, that there were a trend to decreased levels of ADMA over time *P. angulata* L. The difference decreased levels of ADMA day 10, day 31 and day 32, K6 better than K3 ($\Delta 2,4$ ng / mL > $\Delta 2$ ng /mL). Decrease in ADMA levels after administration of verapamil on K6 better than the K3. Average expression DDAH2 the circular and longitudinal position than K3 K6 (18.62% + 2.82% > 16.37% + 6.45%; 16.38% + 2.86% < 18.15% + 6.38%) successive on day 32.

Based on the results, that the visible presence of a trend to decreased levels of ADMA day 10 compared to day 31. The difference decreased levels of ADMA day 32 at K4 better than K1 ($\Delta 2,3$ > $\Delta 1,4$). There were a significant difference in the K1-K4 were shown with p = 0.003, p = 0.006, p = 0.002 at day 10, day 31 and day 32 successive. Average DDAH2 expression showed an increasing trend. In the circular and longitudinal position better than K4 K1 (14.99% + 0.74% > 10.66% + 1.47%; 16.38% + 2.86% < 17.54% + 3.27%) on successive days 32. There was a significant difference in the K1-K4 longitudinal position with a value of significance of P = 0.014.

Group without administration of *P. angulata* L standardized fisalin the accumulation of ADMA levels drop lower than in the group with the provision of *P. angulata* L standardized fisalin. The influence of variations in the levels of ADMA group showed a significant result, as many as 35.5% are influenced by variations in group and 64.5% influenced by other factors. Giving *P. angulata* L standardized fisalin, insulin and verapamil showed a significant difference in the results. Giving *P. angulata* L standardized fisalin can reduce the amount of ROS through its mechanism of action as an antioxidant. In the study conducted Soares et al [16], showed that the seco steroids from *P. angulata* L, as fisalin can improve the situation of NO when induced LPS. Fisalin B is also known to significantly reduce TNF- α , interleukin-6 and interleukin-12.

In another study known mechanism ADMA decrease due to increased enzyme activity in plasma DDAH2. Mechanism of action was consistent with the evidence that overexpression of DDAH-1 in humans showed a decrease in ADMA levels and improving insulin sensitivity [17]. Previous experimental study conducted by Palm et al [18] showed inconsistent results, there was no effect on plasma levels of ADMA DM conditions. Both studies showed a significant relationship that L-arginine may affect the production of NO.

Decreased insulin will lower carbohydrate metabolism resulting in muscle glucose solution. Because of these circumstances will occur reaction conditions for aerobic energy shortages, so that will cause the oxidation of fatty acids in the cell, verapamil is used to balance the condition. Several studies have shown that ADMA is stronger compete penetrate Y + transporter compared with L-arginine, so with a small concentration (1-10 μ g / mL) can reduce NO production significantly. Another theory states that the endothelial cells occurs compartmentalization of amino acids into two compartments. The first part of free arginine in and out through the other amino acids, whereas in the second compartment of the free arginine can not go out while ADMA can enter and interact with NOS. In this second compartment ADMA inhibition ability becomes

larger. This is what can explain that ADMA concentrations are very low only has a great inhibition of the NOS [19].

Asymmetric dimethylarginine known as an inhibitor of eNOS that will affect the production of NO. ADMA acts competitively with L-arginine for binding to the active site of NOS. In several in vitro and in vivo studies suggest that increased ADMA bioavailability NO can trigger the formation of ROS caused. Mechanism of action of L-arginine analogue dimethylated dimethylarginine is symmetric (SDMA) on the NO pathway remains unclear. Most of ADMA (80%) is hydrolyzed by the enzyme DDAH. DDAH enzyme is expressed in two forms, namely DDAH1 and DDAH2 that has the characteristics and distribution as well as encoded by different genes, thus providing different functions. DDAH enzyme 2 is expressed in endothelial cells more than DDAH1 [20].

Effect of *P. angulata* L standardized against expression fisalin DDAH2. Examination of expression DDAH2 conducted to determine the fisalin action on NO synthesis pathway. In hyperglycemic conditions, will improve the regulation of the activity of protein arginine N-methyltransferase (PRMTs) and inhibit the activity of DDAH2. Examination of expression DDAH2 performed on day 32 using the immunohistochemistry method. The examination was conducted on circular and longitudinal position. Quantitatively, the expression can be calculated the percentage using the formula: (number of cells expressing DDAH2 / total number of cells) x 100%. Determined the percentage of cells that expressed the view of the whole kite 40x magnification, then calculations performed at 400x magnification. The amount of protein that is expressed is marked by a color change to brown while the inexpressible stained blue. DDAH expression 2 can be seen in Figure 2.

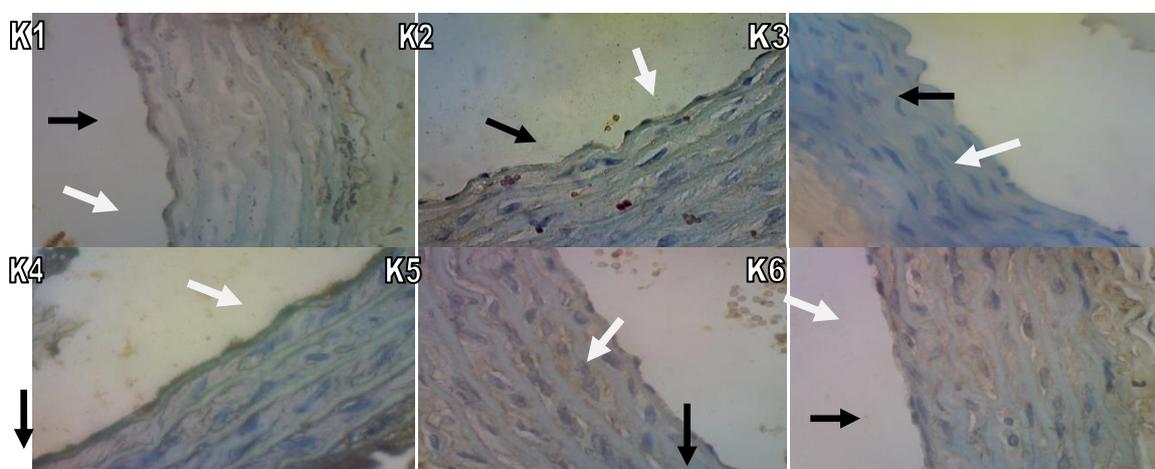


Fig-2: Expression DDAH2 (magnification 400x) (black arrow indicates expression, the white arrow shows no expression DDAH2)

Note:

K1 = distilled water, K2 = distilled water + insulin, K3 = distilled water + verapamil
 K4 = *P. angulata* L standardized fisalin, K5 = *P. angulata* L standardized fisalin + insulin, K6 = *P. angulata* L standardized fisalin + verapamil

Results of analysis of variance (ANOVA) both between and within treatments showed significant results in circular position ($p = 0.033$) and not significant in the longitudinal position ($p = 0.095$). There was a difference between groups and within groups with DDAH2 expression in circular position and there were no difference in longitudinal position. Results of ANOVA analysis on the expression DDAH2 circular position followed by post hoc test - LSD, showed that there were significant differences in the K1-K2 group ($p = 0.041$), K1-K3 ($p = 0.039$), K1-K5 (0,002) and K1 -K6 ($p = 0.006$). Results of ANOVA analysis on longitudinal position DDAH2 expression followed by a post hoc test - LSD, showed that there were significant differences in the K1-K4 group ($p = 0.014$), K3-K4 ($p = 0.010$), K4-K5 (0.026).

Regression analysis showed significant results in circular position ($p = 0.004$; $r = 0.505$ and $R^2 = 0.255$) and no significant results on the longitudinal position ($p = 0.284$; $R = 0.202$ and $R^2 = 0.041$). This means that there were an influence of variation was 25.5% of the group DDAH2 expression in circular position, indicated by the value of r that is. There was no effect on the expression DDAH2 group variation in longitudinal position, indicated by a low value of r .

Percentage expression DDAH2 circular and longitudinal day 32 in all groups can be seen in Figure 3 Comparison of the water extract of herbs ciplukan (*P. angulata* L) along fisalin standardized insulin with insulin alone against DDAH2 will show increased expression of the target action of *P. angulata* L standardized DDAH2 fisalin whether before, after or immediately eNOS.

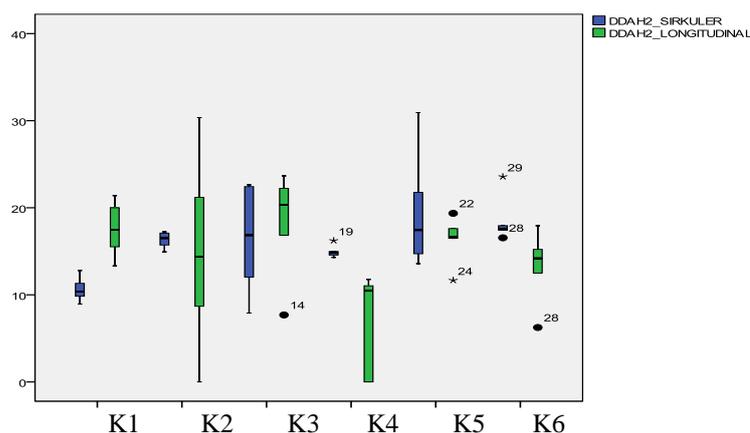


Fig-3: Percentage expression of DDAH circular and longitudinal day 32

Note:

K1 = distilled water, K2 = distilled water + insulin, K3 = distilled water + verapamil
 K4 = *P. angulata* L standardized fisalin, K5 = *P. angulata* L standardized fisalin + insulin, K6 = *P. angulata* L standardized fisalin + verapamil

Group without giving *P. angulata* L standardized fisalin increased expression DDAH2 lower than the group with the provision of *P. angulata* L standardized fisalin the circular group and was not the case in longitudinal position. Effect of variation of the expression DDAH2 circular group showed a significant result, as many as 25.5% were influenced by variations in group and 74.5% influenced by other factors. Giving *P. angulata* L standardized fisalin, insulin and verapamil showed a significant difference in the results.

Increased levels of NO are not only influenced by antioxidant activity alone. But according to Sydow increased production of NO may be due to increased endogenous inhibitor of eNOS that asymmetric dimethylarginine (ADMA) [17]. Increased superoxide production in diabetes will increase regulation of the activity of protein arginine N-Methyltransferase (PRMTs) and inhibit the activity of dimethylaminohydrolase dimethylarginin (DDAH). Increased concentrations of ADMA inhibits eNOS in producing NO.

Research conducted in cell culture showed that the decrease in blood sugar levels, ox LDL, cholesterol, and homosistin associated with decreased activity of DDAH. Decreased DDAH activity is also influenced by oxidative stress in vascular endothelial cells, which is caused by the presence of TNF. The addition of antioxidants are known to have a positive effect on reducing the activity of DDAH, ADMA accumulation and increased production of NO [21]. This is in line with research conducted by Vallance et al which states that the oxidant-sensitive enzyme DDAH is caused by the presence of the group in the catalytic sulfhidridil [19].

Increased activity and expression DDAH2 in this study was associated with reduced accumulation of ADMA. This is in line with research conducted Arigoni et al, DDAH2 found increased expression consistent with the presence of elevated levels of ADMA, whereas decreased expression DDAH2 possible will contribute to increased levels of ADMA[22]. DDAH2 possible decrease in activity as the cause of increased levels of local ADMA ADMA levels that do not affect the circulation. DDAH2 induction occurs when increased levels of ADMA straight downhill. This is the underlying some other research to develop pharmacological therapies that can inhibit DDAH activity that will affect the vasoconstriction and NO production.

CONCLUSION

P. angulata L standardized fisalin can reduce levels of ADMA and increase the expression of DDAH2. *P. angulata* L standardized fisalin can improve NO synthesis through a decrease in ADMA levels and increased expression of DDAH2.

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CONFLICTS OF INTEREST

Authors declare no conflicts related to the research and publication of the results of this research.

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