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Effects of Ethanol Extract of *Rosa damascene* on HbA1c Level and NF-κB Expression in Diabetic Rats

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Abstract

Diabetes mellitus (DM) is a chronic hyperglycemic that can cause complications in several organs. It could lead glycosylated hemoglobin or HbA1c formation which has ability undergo further changes into Advanced Glycation End Products (AGEs) and stimulate activation of transcription factors such as nuclear factor kappa B (NF-κB). Some studies showed that anthocyanin has antioxidant and anti-inflammatory activity and it has been found *Rosa damascene* contain high level of anthocyanin. Total anthocyanin content was 0.459 ± 0.003 mg/L. The aim of this study was to investigate inhibitory effect of *R. damascena* extract on HbA1c levels and NF-κB activation in diabetic rats. Male wistar rats (n=24) were divided into 6 groups as normal control (KN), streptozotocin-induced diabetic control (KP), diabetic treated with *R. damascene* (P1, P2, P3; 250, 500 and 1000 mg/kg/d respectively) and diabetic treated with metformin (KM; 500 mg/kg/d). This was carried out for 28 days. The inhibition mode of *R. damascene* extract was examined by measuring HbA1c levels and expression of NFκB by immunohistochemistry. The results showed p values > 0.05 for HbA1c and p < 0.05 for NFκB. From immunohistochemical staining, it seen the expression of NFκB was low in treated group (P1, P2, P3 and KM) compared with KP. Thus, oral administration of *R. damascene* extract for 28 days could not reduce HbA1c levels, but can supress NFκB expression.

Keywords

Diabetes mellitus, *Rosa damascene*, HbA1c level, NFκB expression.

INTRODUCTION

Diabetes mellitus or DM is a chronic hyperglycemic that can cause complications in several organs including coronary artery disease, cataracts, renal failure and stroke (1). Globally, an estimated 422 million adults had diabetes in 2014, compared to 180 million in 1980. This escalation was associated with an increase in risk factors including overweight or obese. In Indonesia, enhancement Diabetes mellitus started around 4% in 1980 and has been increased to 7.5% in 2014 (2). Compared to 2013, there was an increase Diabetes Mellitus prevalence of 2% in population ≥ 15 years in 2018 (3).

Glycosylated hemoglobin or HbA1c examination is one of the criteria for diagnosing Diabetes Mellitus. Diabetes Mellitus is established if HbA1c level ≥ 6.5% by the method standardized by the National Glycohaemoglobin Standardization Program (NGSP) (4). Rate Glycosylated hemoglobin reflects the blood sugar level during the previous 2-3 months.

Diabetes Mellitus could lead glycosylated hemoglobin or HbA1c formation which has ability undergo further changes into Advanced Glycation End Products (AGEs) (5). The binding of AGEs or other ligands to RAGE (Receptor for Advanced Glycation End Product) can stimulate various signal transduction cascades leading to formation of ROS and activation of transcription factors such as nuclear factor kappa B (NF-κB). Increased production of ROS has been linked to impaired endothelial function and structural damage in several macromolecules including carbohydrates, protein, lipid and DNA (6). Nuclear Factor kappa B or NF-κB plays an important role in the pathogenesis of complications of diabetes mellitus. Activation of NF-B will trigger the emergence of various inflammatory mediators such as cytokines, chemokines, and cell adhesion molecules. Overexpression of NF-κB TNF-α, interleukins, TGF-β, Bcl2 and other proinflammatory proteins and pro-apoptotic genes is the key to the development of vascular complications in diabetes. In addition, the high expression of NF-B will cause vascular calcification (7).

Rosa damascene is one of the ornamental plants and is cultivated extensively around the world, including Indonesia. Badan Pusat Statistik (BPS) stated that the harvested area of Rosa damascena ranks second after chrysanthemum was 3,723,228 m^2. While the province of East Java ranks first for the production of roses (8). In addition to various commercial products, rosa damascena has other benefits as anti-cancer, antimicrobials, relaxants and anti-depressants, antioxidant, analgesic, and anti-inflammatory. It is the most important plant from the Rosacea family and has received less attention to therapeutic application (9). Furthermore will be discussed regarding the effect of *R.*

damascene to improve one of the chronic diseases, diabetes.

Saptarini *et al*. (10) compared total content between *R. damascene* and China Red Rose which extracted by maceration procedure. It was found that total anthocyanin content of *R. damascene* and China Red Rose was 0.459 ± 0.003 mg/L and 0.186 ± 0.006 mg/L, respectively. Anthocyanins are a group of water-soluble pigments and widely distributed in colorful fruits and vegetables such as berries, cherries, hawthorns, peaches, grapes, apples, red onions, red radishes, black beans, eggplants, red cabbage, and purple sweet potatoes (11).

The antioxidant activity of anthocyanins is influenced by the structure of the anthocyanins, the number and arrangement of groups aromatic hydroxyl, presence of electron-donating properties and electron withdrawal in the ring structure. This causes anthocyanins to be highly effective hydrogen donor for reactive free radicals thereby preventing the formation of further radicals (12).

Previous study stated that anthocyanins have antioxidant properties and anti-inflammatory activity by inhibiting the expression of NFκB pathway induced by palmitic acid in dose dependent way (13). Administration of cyanidin-3-glucoside (20 mg/kg/day for 12 weeks) in db/db mice, proved can reduce body weight, glucose levels, HbA1c, C-peptide, serum insulin, blood pressure, and several parameters related to kidney damage (serum creatinine, urinary albumin, BUN, albumin/creatinine ratio, fibronectin, collagen IV, TGF 1, MMP9, TNF-α, monocyte chemotactic protein-1, IL-1 and α-smooth muscle actin expression) (14). In another study in vitro on visceral adipose tissue, administration of protocateccuic acid (PCA) can reduce the activity of protein-tyrosine phosphatase 1B, phospho-p65 NF-κB and IL-6 (15). We thus investigated whether supplementation of *R. damascene* extract inhibits the HbA1c formation and NF-κB activation in diabetic rats.

MATERIALS AND METHODS

Plant

R. damascene were obtained from Bumi Aji sub-district, Batu City. *R. damascene* extract was done by maceration. It washed and dried at a temperature of 32-35^{0}C (avoid direct sunlight). Furthermore, the dried petals were blended and macerated with 96% ethanol food grade for 24 hours. The mixture was evaporated to obtain extract.

For the dosages, researcher followed Gholamhoseinian *et al*. (16) which using red rose extract at a dose of 100 mg/kgbw/d, 250 mg/kgbw/d, 500 mg/kgbw/d and 1000 mg/kgbw/d. It had been reported that *R. damascena* in methanol extract was able to reduce sugar levels blood significantly compared to controls. Therefore, in this

research, researcher will using a dose of *Rosa damascena* extract at a dose of 250 mg/kgbw/d, 500 mg/kgbw/d and 1,000 mg/kgbw/d.

Animals

Twenty-four male *Rattus norvegicus* (8-10 weeks; weighing 150-200 gram) have been used for experiment. They were caged and kept under normal laboratory condition. Rats were classified into 6 groups, within 4 rats in each group. Group KN was a normal rat, group KP was a diabetic rat, animal from group P1, P2 and P3 received doses 250, 500 and 1,000 mg/kg/d of *R. damascene* extract, and group KM was treated with metformin (500 mg/kg/d) by gavage. Metformin is a first-line drug for diabetes and effective as monotherapy or in combination with other antidiabetics (17). *R. damascene* extract and metformin were dissolved into distillated water and administered orally by intra-gastric route (in a final volume 1 mL). All studies procedure and method have been approved by Health Reasearch Ethical Clereance Commission of Universitas Airlangga Faculty of Dental Medicine with a number 080/HRECC.FODM/II/2021.

Location and Time

Rats were treated in the Pharmacology laboratory of the Faculty of Medicine, Airlangga University Surabaya. Examination of HbA1c level were carried out at the Central Health Laboratory Surabaya. Meanwhile, immunohistochemical examination was carried out in the laboratory biochemistry Universitas Brawijaya Malang. This research was conducted for 3 months.

Experimental design

Rats (n = 24) devided into 6 groups (KN, KP, P1, P2, P3 and KM) and have been acclimatizated for 7 days. They were placed in cages with well ventilation and cared under standardized eviromental circumstances (22-28°C, 12 h dark light cycle and free access to food and water). For induction of diabetes, 50 mg/kg streptozotocin (STZ) was injected in rats (all groups, except KN), intraperitoneally. Five days after streptozotocin administration, rats blood glucose levels were checked in the tail vein with a glucometer. Sugar levels above 200 mg/dl were declared as hyperglycemia and included in inclusion criteria of study. KN and KP were received normal feed and water ad libitum, while P1, P2, and P3 were treated with *R. damascene* extract with doses of 250 mg/kgbw/d, 500 mg/kgbw/d, and 1,000 mg/kgbw/d, respectively. Then, group KM was administrated metformin 500 mg/kgbw/d. Administration of *R. damascena* extract and metformin was fixed for 28 days. After the period was over, the rats were weighed and blood sugar levels were checked with glucometer. Rats were euthanized and blood samples were collected for HbA1c levels and aorta was excised for NF-κB expression by immunohistochemistry.

HbA1c levels

Levels of HbA1c were measured using glycohemoglobin A1c assay kit (classification No:30168000, The Pharmaceuticals, Medical evices and Other Therapeutic Product act of Japan). Samples were sentrifuged (800 g for 5 minutes), then collect 25 µL from the bloodcell layer and add it into pretreatment solution, and mix. Measure this mixture with an automated Analyzer and read the absorbance. The measurement need to be converted into NGSP values (%).

Immunohistochemical Analysis of NF-κB Expression

Paraffin block were cut into 3-5 µm thick and attached to a glass slide was fixed with acetone for 2-3 minutes and stored at 4˚C. fixing the slide with methanol and allowed to stand for 5 minutes, then dried, aerated and washed with Phosphate Buffer Saline (PBS) pH 7.4 for 5 minutes with three repetitions. Added H_2O_2 for 10 minutes and washed with PBS for 5 minutes with three repetitions. FBS (Fetal bovine serum) containing 0.25% Triton X-100 was added and incubated for 1 hour at room temperature for blocking process. Furthermore, slide washed with PBS for 5 minutes with three repetitions. Primary antibody (monoclonal anti p65) was added and slide was incubated for 24 hours and washed with PBS for 5 minutes with three repetitions. Then secondary antibody was dripped and incubated for one hour and washed with PBS for 5 minutes with three repetitions. The next step was addition of SA-HRP for 40 minutes (washed with PBS for 5 minutes with three repetitions), and dripped with DAB for 5-30 minutes. Then counterstain with Mayer's hematoxylin for 10 minutes, then rinsed and washed with distilled water for 10 minutes, dried and covered with cover glass (18). Slides were examined under microscope and cells expressing NF-κB (there was a color change to brown) was calculated with a magnification of 100 x. This calculation is carried out in 20 fields of view and divides the number obtained by 20 to get the average.

Statistical analysis

Saphiro-Wilk and Levene test were performed for normality and homogeneity of data ($p>0.05$). For the data normally distributed and homogenous could be followed with One Way ANOVA test ($p<0.05$) and LSD test was used to compare between groups. If the data did not confirm homogenity of variances, Games-Howell tests can be performed to see differences between groups. If the data does not meet one of the requirements for parametric analysis, non-parametric statistical tests can be performed using the Kruskal-Wallis test, then continued with the Mann-Whittney with confidence level 95% ($\alpha = 0.05$) to see the difference between treatment group.

RESULTS

Effect of *Rosa damascene* on HbA1c level

The average KP had a lower mean HbA1c value than the treatment rat group. Based on the results of the normality test, it can be seen that the p value > 0.05. Data analysis was continued by looking for homogenity. The Lavene test showed p value < 0.05, which means data was not homogeneous. Furthemore, Brown-Forshyte test was conducted and p < 0.05 was obtained. Thus, H0 was rejected and H1 is accepted or there was a difference between the control and treatment groups. In this study, the Games-Howell post hoc test was carried out because the variation in the study sample population was not homogeneous. There was a significant difference between KN and treatment groups (P2, P3 and KM (p < 0.05)). Whereas, no significant difference was found between KP and other groups KN, P1, P2, P3 and KM (p > 0.05) (Figure 1).

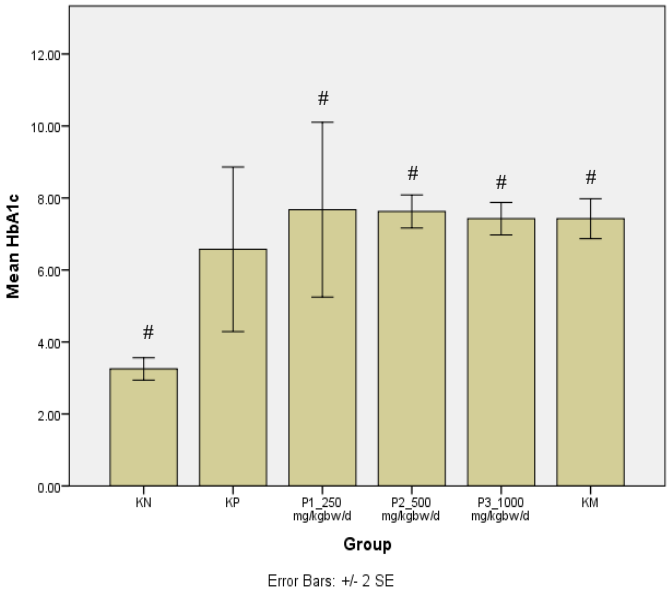

Figure 1. Comparison of mean of HbA1c. Values marked with # showed no difference (p >0.05) compared to positive controls (KP)

Effect of *Rosa damascene* on NFκB level

The Shapiro-Wilk test showed p > 0.05 and Lavene test of the NFκB data was p = 0.062 (p > 0.05). Furthermore, one way Anova test was performed, the p value was 0.00 (p < 0.05), therefore, Tukey-HSD method was used to see differences between groups. Based on the Tukey-HSD test, p value < 0.05 was found between KN and KP; P1. In addition, there were also significant differences between KP and KN; P2; P3; KM (Figure 2). Differences in NFκB expression between groups by immunohistochemistry were shown in Figure 3.

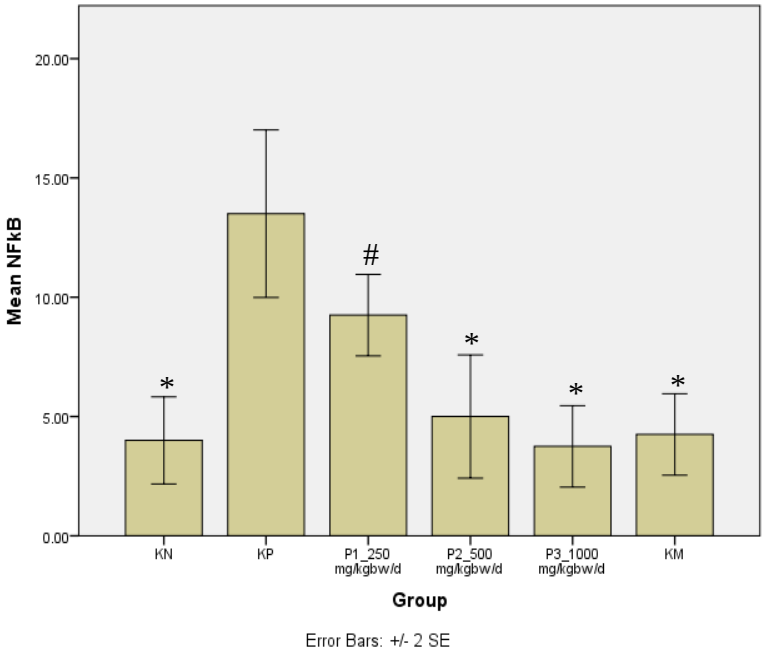

Figure 2. Mean comparison of the NFκB expressions. Values marked with *were significantly different ($p<0.05$) compared to KP. While # was not significantly ($p>0.05$) different to KP

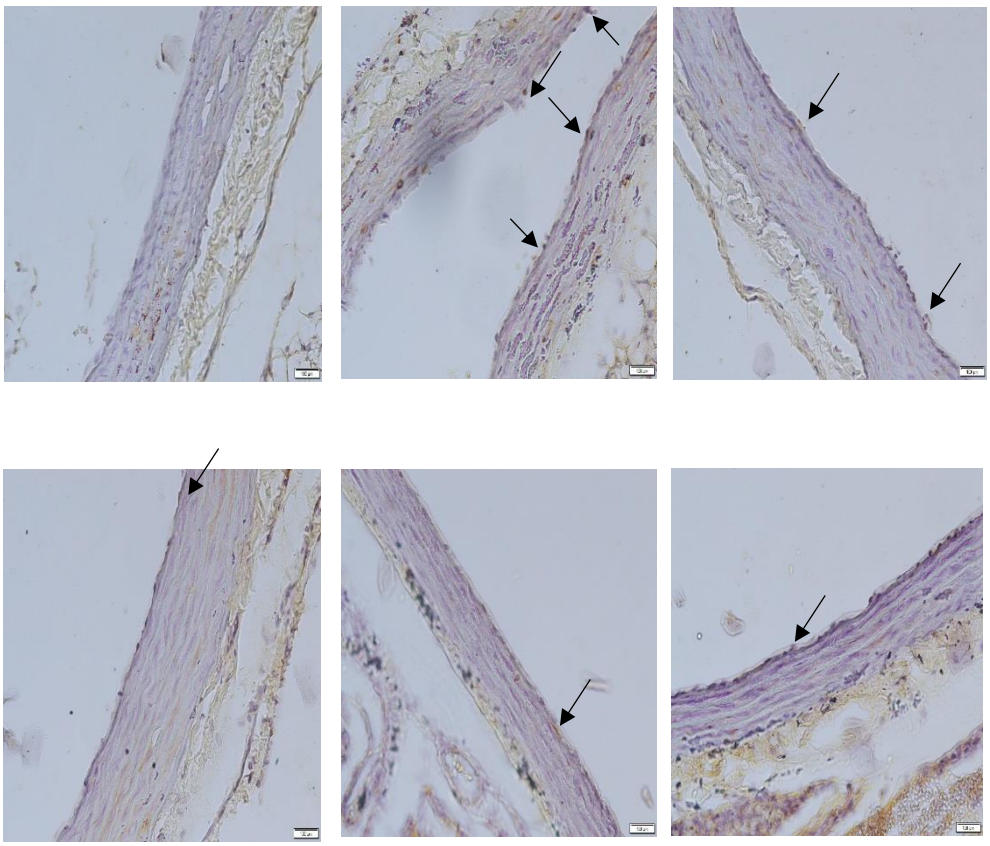

Figure 3. Immunohistochemical comparison between KN, KP, P1, P2, P3 and KM groups. In the KN group, it was seen few expressions of NFκB compared to KP (black arrow). While in the treatment group there were decreasion NFκB expression in P3 and KM. (400x magnification)

DISCUSSION

Saptarini *et al*. (9), compared total content between *R. damascene* and China Red Rose which extracted by maceration procedure. It was found that total anthocyanin content of *R. damascene* and China Red Rose was 0.459 ± 0.003 mg/L and 0.186 ± 0.006 mg/L, respectively. Anthocyanin have several benefits for diabetic patients, including reducing fasting blood sugar, serum insulin, triglyceride levels, cholesterol, HbA1c, increasing insulin secretion and insulin sensitivity (19). Anthocyanins influence expression gen of GLUT4 and its translocation, enhance PPARγ activation in skeletal muscle and adipose tissue, enhance AMP-activated protein kinase activation, incerase adiponectin and leptin, and, anthocyanins also inhibit secretion of α-glucosidase from intestine and α-amylase from pancreas (20).

In this study, average HbA1c in KN group was low at 3.25%, while in the KP was 6.6% (data not shown). Furthermore, KM group obtained a value of 7.4%. Furthermore, P1, P2, and P3, HbA1c values was 7.7%, 7.6%, and 7.4%, respectively. After statistical tests were carried out, data showed $p > 0.05$. so that it did not show any effect of *R. damascene* on HbA1c levels in diabetic rats.

Streptozotocin has been known as an agent that causes an increase free radicals and damage to pancreatic beta cells to cause hyperglycemia. Furthermore, this condition is an important key for diabetes mellitus complications. The role of antioxidants is very important to break the chain of radical reactions. Antioxidant enzymes that functions to oxidize oxidant molecules is superoxide dismutase (SOD) (converting superoxide radicals to hydrogen peroxide), GSH-peroxidase (GPx) and catalase (CAT) which convert hydrogen peroxide into water and oxygen. Some previous studies reported inconsistent results on SOD and catalase activity in various tissues. It could influence by differentiation in expression of antioxidant enzymes as well as techniques for producing rat models of diabetes mellitus (21). This mechanism may underlie the low HbA1c in KP and P1.

Pelargonidin administration in rats induced by streptozotocin for 5 weeks showed a decrease in HbA1c levels (22). Qin *et al*. (14) also reported that a decrease in HbA1c was observed in db/db mice given C3G for 12 weeks of therapy. Falah *et al*. (23) concluded that the consumption of anthocyanins will provide benefits on fasting blood sugar levels and 2 hours pp, HbA1c and HOMA-IR with a duration of > 8 weeks and with a dose of > 300 mg/day. Contrary to this, in this study, the average increase in HbA1c was 6.6% in positive control group (KP) or diabetes group. The average value is lower than treatment groups (P1, P2, and P3), which ranged from 7.4 to 7.7%. It can be

concluded that statistically administration of *R. damascene* to rats model diabetes mellitus cannot reduce HbA1c levels or not showed significant differences between groups.

Previous Study by Yang *et al*. (20) showed there was an escalation in the number of SOD and decrease in the enzyme catalase in brain tissue at the 8th week post-induction with STZ. Meanwhile, at 16th week after STZ induction, there were reduction of SOD and catalase activity in the cortex, hippocampus and blood vessels. Another study conducted for 2 weeks it was mentioned that there was an insignificant expression of SOD in rats control and diabetic mice. In addition, HO-1 mRNA expression was increased in group of diabetic rats compared with a group of healthy rats (24).

Several previous studies stated that experimental research for diabetes mellitus showed inconsistent results in SOD and catalase activity in various tissues. Some of the factors that causes of this include differences in the expression of antioxidant enzymes as well as technique to generate a rat model of diabetes mellitus (20). These mechanisms may underlie low level of HbA1c on KP and P1.

Meanwhile, studies conducted on humans did not show consistent results. Administration of capsules containing concentrated powder of juice cranberries for 12 weeks did not show significant changes in levels of fasting blood sugar, HbA1c, fructosamine, triglycerides and HDL or LDL levels between the treatment and placebo groups (25). Another study reported that sour cherry juice given to female patients with Type 2 diabetes can reduce body weight, blood pressure and HbA1c after 6 weeks (26).

Based on the explanation above, giving *R. damascene* did not have an effect on HbA1c levels and probably caused by duration of study was relatively short. Besides, it needs others investigation for endogenous antioxidants level from rats thought to play role in low levels of HbA1c in the positive control group compared with treatment group.

Nucleus Factor kappa B or NFκB is protein transcription factors involved in cellular responses to various stimuli, including stress, cytokines, free radicals, bacterial and viral antigens. This factor is present in all types of tissue cells and is involved in various cellular responses (26). NFκB activation can lead to increased expression of pro-inflammatory and pro-apoptotic genes as well as associated with an increased incidence of complications in diabetes mellitus (27).

NFκB contain homo or heterodimer protein (p65 or RelA, p50/p105, c-Rel, p52/p100 and Rel B). NFκB forms a complex with inhibitory protein IκB. Degradation of IκB inhibitor protein lead NFκB translocate to the nucleus and bind to DNA and induce

transcription of proinflammatory genes. Several studies have shown that the anthocyanidin delfinidine can inhibit the degradation of the inhibitory IκB protein and the translocation of p65 into the nucleus. In addition, the anthocyanin is also the most potent COX-2 inhibitor, both at the mRNA and protein levels, suppressing LPS which stimulates the activation of other transcription factors, namely C/EBP, AP-1, however, not CREB (11).

Paixao *et al*. (28) reported that malvidin-3-glucoside can suppress NFκB inhibition on arterial endothelial cells from bovine. In addition, malvidin can inhibit p65 which is one of the subunits of NFκB. In vitro studies on visceral adipose tissue (VAT) with protocatechuic acid could reduce the activity of PTP1B, phosphor-p65, NFκB and IL-6 in VAT from obese subjects (15).

In addition, in vitro study used HK-2 cells treated with pure C3G, showed the presence of increased cholesterol efflux, ABCA1 expression, PPARα, LXRα, decreased proinflammatory molecules such as ICAM1, MCP1, TGFβ1, and NFκB (29). Furthermore, administration of Medox capsules containing pure anthocyanins isolated from bilberry and blackcurrent in human monocyte cultured cells and healthy human subjects (for 3 weeks) showed direct inhibition of NFκB transactivation and decreased concentrations of proinflammatory, chemokines and NFκB-regulated immunoregulatory cytokines (30) In addition, hypercholesterolemic subjects who were given mixed anthocyanin purified (320 mg/day) for 24 weeks showed a decrease in NFκB-associated inflammatory response (31).

This study showed that administration of *R. damascene* could reduce NFκB expression in the rat aorta ($p < 0.05$). It can be seen from the immunohistochemical results, the cell count expressing NFκB in the rat aorta decreased with the administration of *R. damascene* extract. This is in accordance with several previous studies.

CONCLUSIONS

We observed that Extract *R. damascene* has potentially effect to improve NFκB in diabetic rats, but need further some investigation about endogen antioxidant and duration of study to prove effect of *R. damascene* to HbA1c level.

AUTHOR CONTRIBUTIONS

Choirotussanijjah: conceptualization, methodology, writing – original. Harianto Notopuro: supervision, conceptualization, validation. Ema Qurnianingsih: supervision, reviewing and validation.

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The authors declare no conflict of interest.

CONFLICT OF INTEREST

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