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Hypoglycemic and Antioxidative Effects of Pomegranate (*Punica granatum* L.) Juice in Streptozotocin Induced Diabetic Rats

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Abstract: Diabetes mellitus is a metabolic disorder that is characterized by hyperglycemia and associated with increased oxidative stress. The polyphenol property in pomegranate juice was reported to have significant antioxidant effect. The present study aims to investigate the hypoglycemic and antioxidative effect of pomegranate juice in streptozotocin-induced diabetic rats. Twenty healthy male *rattus norvegicus* with age of eight weeks and weighing at average of 200 g were randomly assigned into four groups. Diabetic models were induced by using 60 mg/kg body weight (BW) streptozotocin (STZ) intraperitoneally. The rats were allocated into four groups, group treated with 0.1 mg/kg BW glibenclamide, groups supplemented with 1, 2 and 4 mL/200 g BW pomegranate juice, respectively. Evaluation of hypoglycemic and antioxidative effect were performed by measuring blood glucose level, lipid peroxidation, including malondialdehyde (MDA) and advanced glycation end-product (AGE) before and after treatment. Lipid profile was also measured before and after treatment. Treatment with glibenclamide and supplementation with three different doses of pomegranate juice in diabetic rats were significantly reduced blood glucose level. Supplementation with 2 mL/200 g BW pomegranate juice was significantly reduced MDA concentration, whereas the AGE concentration was not significantly decreased by pomegranate juice supplementation. Lipid profile related to total cholesterol and triglyceride levels were not significantly reduced. Furthermore, high density lipoprotein was not increased significantly after each treatment. Pomegranate juice has hypoglycemic and antioxidative effects in streptozotocin-induced diabetic rats.

Key words: Pomegranate juice, diabetes mellitus, antioxidant, AGE, malondialdehyde, metabolic disorder, hyperglycemia

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia a result of defective insulin secretion, insulin action resistance, or both (ADA, 2012). The prevalence of DM is expected to reach up to 4.4% in the world by 2030 (Wild *et al*., 2004). Hyperglycemia in DM causes excessive free radical production (Brownlee, 2001). Free radical over production will lead to increase in oxidative stress and subsequent auto-oxidation of glucose, amino acid and lipid (Marritim *et al*., 2003). Advanced glycation endproducts (AGEs) are a group of modified molecular species formed by non enzymatic reactions of reducing sugars with proteins, lipids or nucleic acids. AGEs are generated in hyperglycemia and their accumulation is accelerated in diabetes (Lautenslager *et al*., 2011; Schalkwijk and Miyata, 2012). Accumulation of AGE increases oxidative stress in various tissues, leading to several pathologies such as atherosclerosis, diabetic nephropathy, diabetic retinopathy and cataract (Droge, 2002; Ueno *et al*., 2000).

The tissue damage due to AGE accumulation is mediated by modification of intracellular protein, extracellular matrix and plasma protein (Shinohara *et al*., 1998; Charonis *et al*., 1990). The changes in plasma protein cause AGE binding into its receptors and inflammatory cytokine and growth factor released that disturb vascular integrity (Li *et al*., 1996). Subsequently, increased of AGE production is able to disrupt nitric oxide bioavailability and cause endothel dysfunction. Cytotoxic effect of free radicals to vascular endothelial cells decreases the bioavailability of nitric oxide (NO), produced by endothelial NO synthase (eNOS). The NO impairment implicates in atherosclerosis formation (Ignarro *et al*., 2006). Free fatty acid (FFA) levels, which are elevated in diabetes and insulin resistance, may also contribute to the increased production of reactive oxygen species (ROS) due to increased mitochondrial uncoupling and beta-oxidation (King and Loeken, 2004; Bikopoulos *et al*., 2008). Several studies have shown that antioxidants could be useful in preventing or attenuating the adverse effects of chronic hyperglycemia (Omar *et al*., 2011; Sivakumar *et al*., 2010; Cemek *et al*., 2008).

World Health Organization (WHO) recommended for the assessment of traditional medicinal plant in connection with the management of DM. Now a days herbal treatments are becoming increasing by popular as the

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herbal preparations have no or least side effects (Rajasekaran *et al*., 2001). Currently available therapies for DM include insulin and various oral antidiabetic agents such as sulfonyl urea, biguanides, di-peptidyl peptidase-4 inhibitor and thiazolidinediones, which are used as monotherapy or in combination to achieve better glycemic regulation (Jarald and Joshi, 2008; Gy *et al*., 2005). Many oral hypoglycaemic agents have significant side effects (Rang *et al*., 1991), some are in effective in chronic diabetic patients (Pari and Saravanan, 2004) and therefore, there is a need to find safer and more effective antidiabetic drugs (Grover *et al*., 2002). Thus, there is an increasing demand of new antidiabetic natural products with lesser side effects and high antidiabetic effects (De *et al*., 2011).

The traditional medicines demonstrate a bright future in diabetic therapy and enhance the importance application of traditional herbs. Several plants have been used in medical therapy due to their effect in reducing free radical excess. These plants contain flavonoid which acts as antioxidant mediated by their free radical scavenging properties and their ability to reduce the formation of free radicals (Pietta, 2000). One of the potential agents for future diabetic therapy is Pomegranate that contains flavonoid, proantosianidin, ellagitanin and gallotanin. They are proven to able to protect pancreatic beta-cells from free radicals damage (Elfalleh *et al*., 2011; Khalil, 2004). Previous study by Mara *et al*. (2000) reported that antioxidant activity of pomegranate was three times higher than red wine and green tea. Pomegranate juice possesses significant greater antioxidant capacity at lower concentrations than other grape or blueberry juice. The pomegranate and its extracts have powerful antioxidant effects, which have also been revealed for other fruit juices, including grape, blackberry and branberry juice and in green tea (Gil *et al*., 2000). The higher antioxidant activity is facilitated by high anthocyanin flavonoid and total flavonoid content in pomegranate juice compare to other juices (Ignarro *et al*., 2006).

Pomegranate is rich in antioxidant of polyphenolic class which includes tannins and anthocynins and flavonoids (de Nigris *et al*., 2007; Ricci *et al*., 2006).

Collectively, the present study was aimed to investigate the hypoglycemic and antioxidant effect of pomegranate juice in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Experimental animals: The animals used in this study were eight week old-male *Rattus norve*gicus and weighing at average of 200 g. The pomegranate fruit were obtained from local market in Jakarta, Indonesia. The study was approved by Ethical Commitee, Faculty of Medicine, Universitas Gadjah Mada.

Juice preparation: Pomegranate (*Punica granatum*) fruits were washed and manually peeled, without separating the seeds. Juice was obtained using a commercial juicer.

Induction of experimental diabetes: Diabetes rats were induced by *intraperitoneal* injection of STZ (nacalai) at dose of 60 mg/kg body weight (BW) in 0.1 M citrate buffer, pH 4.5 according to a method described by Ganda *et al*. (1976). Diabetic condition in rat was confirmed 48 h after injection with STZ by measuring 10 h fasting blood glucose level taken from *retro orbitalis plexus*. Animals with blood glucose level above 280 mg/dL were considered to be diabetic and included in the experiment (Kanter *et al*., 2006).

Experimental design: Twenty diabetic rats were randomly assigned into four groups of equal number and weight (five animals each) as follow:

Group I: Diabetic rats treated with standard drug, i.e., glibenclamide (0.1 mg/kg BW, orally) daily within four weeks
Group II: Diabetic rats treated with pomegranate juice (1 mL/200 g BW, orally) daily within four weeks
Group III: Diabetic rats treated with pomegranate juice (2 mL/200 g BW, orally) daily within four weeks
Group IV: Diabetic rats treated with pomegranate juice (4 mL/200 g BW, orally) daily within four weeks

At the beginning and the end of the experimental (four weeks), blood samples were collected from *retro orbitalis plexus* after the animals had been fasted for 10 hours. For serum separation, blood samples were incubated at room temperature to get the blood clot and then centrifuged for 15 min at 3000 rpm. Serum was carefully aspired using a needle and transferred into dry clean test tubes and kept frozen at -20°C until chemical analysis.

Blood glucose, MDA, AGE and lipid profile: The blood glucose level determination was carried by GOD-PAP methods (Diasys Diagnostic) based on glucose oxidase. Serum concentration of malondialdehyde (MDA) was determined with TBARS methods using spectrophotometer at 510, 532 and 560 nm. Serum concentration of AGE were determined using ELISA kit (HRD) and analyzed by microplate reader at 450 nm. Serum concentration of total cholesterol, triglyceride and HDL were determined using enzymatic methods (DiaSys Diagnostic).and absorbance was measured using spectrophotometer at 546 nm.

Statistical analysis: All data were presented as mean±SE. All data were tested for normal distribution by Shapiro-Wilk test. Significant differences between before and after treatment were analyzed with Mann-Whitney test. Significant differences among the experimental groups were determined by one-way analysis of variance or Kruskal-Wallis test based on data distribution. The analysis was performed using the

Table 1: Baseline parameter in each group before treatment

Parameter	Group I (Mean±SE)	Group II (Mean±SE)	Group III (Mean±SE)	Group IV (Mean±SE)
Glucose (mg/dL)	394.50±156.84	479.62±164.70	415.75±78.92	397.89±214.80
MDA (µmol/L)	3.45±2.62	2.02±1.68	2.77±1.95	3.16±2.07
AGE (µmol/ml)	400.05±9.75	258.42±82.15	316.73±93.45	336.97±348.09
Total cholesterol (mg/dL)	67.24±27.39	67.24±27.39	60.64±21.98	92.58±41.82
Triglyceride (mg/dL)	378.30±328.21	231.03±229.13	368.30±321.67	334.38±628.55
HDL (mg/dL)	46.47±8.86	42.93±6.61	41.90±2.95	49.84±4.29

I, II,III and IV: Diabetic rat was treated with glibenclamide 0.1 mg/kg BW, pomegranate juice 1, 2 and 4 mL/200 g BW, respectively

Table 2: Concentration of glucose, MDA, AGE and lipid profile before and after treatment

Group	Before	After	p-value*	Difference	p-value**
Glucose, mean±SE					
Group I	394.50±156.84	149.73±93.27	<0.05	-244.77±111.08	>0.05
Group II	479.62±164.70	130.65±100.59	<0.05	-348.97±227.90	-
Group III	415.75±78.92	82.36±39.04	<0.05	-333.39±108.96	-
Group IV	397.89±214.80	144.14±174.97	<0.05	-253.74±190.49	-
MDA, mean±SE					
Group I	3.45±2.62	0.71±0.24	>0.05	-2.74±2.49	>0.05
Group II	2.02±1.68	0.69±0.22	>0.05	-1.32±1.50	-
Group III	2.77±1.95	0.68±0.38	<0.05	-2.08±1.67	-
Group IV	3.16±2.09	0.85±0.12	>0.05	-2.31±2.08	-
AGE, mean±SE					
Group I	400.05±9.75	156.00±139.72	>0.05	-243.48±157.07	>0.05
Group II	258.42±82.15	236.63±149.18	>0.05	-21.79±193.02	-
Group III	316.73±93.45	219.00±193.83	>0.05	-96.96±251.79	-
Group IV	336.97±348.09	126.26±160.58	>0.05	-210.70±439.09	-
Total cholesterol, mean±SE					
Group I	67.24±27.39	66.17±20.57	>0.05	-1.06±19.16	>0.05
Group II	70.70±24.15	49.11±22.68	>0.05	-21.58±30.88	-
Group III	60.64±21.98	41.69±15.61	>0.05	-18.95±28.08	-
Group IV	92.58±41.82	57.63±9.15	>0.05	-34.94±39.96	-
Triglyceride, mean±SE					
Group I	378.30±328.21	60.46±34.02	>0.05	-317.84±350.65	>0.05
Group II	231.03±229.13	49.99±29.05	>0.05	-181.15±242.03	-
Group III	368.30±321.67	44.39±24.29	>0.05	-323.91±341.82	-
Group IV	334.38±628.55	69.24±29.95	>0.05	-265.13±599.69	-
HDL, mean±SE					
Group I	46.47±8.86	46.62±6.09	>0.05	0.15±9.32	>0.05
Group II	42.93±6.61	47.58±4.02	>0.05	4.65±8.67	-
Group III	41.90±2.95	46.06±5.72	>0.05	4.15±6.43	-
Group IV	49.84±4.29	52.30±10.26	>0.05	2.46±12.76	-

*p-value from Mann-Whitney test between before and after treatment
**p-value from Kruskal-Wallis test of differences among groups
Group I, II, III and IV: Diabetic rat was treated with glibenclamide 0.1 mg/kg BW, pomegranate juice 1, 2 and 4 mL/200 g BW respectively

SPSS statistical program (SPSS Inc, USA). Statistical significance was considered at p value<0.05.

RESULTS

Blood glucose level, MDA and AGE concentration as well as lipid parameter were comparable in the baseline study as shown in Table 1.

Blood glucose level was significantly reduced after 4 weeks treatment with glibenclamide and the three different doses of pomegranate juice. Of our interest, diabetic rats treated with three different doses of pomegranate juice had the same effect in decreased blood glucose level compared with those treated with glibenclamide, as shown in Table 2. Furthermore, the level of blood glucose reduction was comparable between glibenclamide treatment and three doses of pomegranate.

Serum MDA level was significantly decreased after four weeks treatment with 2 mL/200 g BW pomegranate juice. However, other group treated with glibenclamide or other doses of pomegranate juice (1 and 4 mL/200 g BW) showed a tendency toward reduced MDA level. The level of MDA reduction was not significantly different between glibenclamide treatment and pomegranate juice (Table 2).

Serum concentrations of AGE were not reduced significantly after glibenclamide treatment. Subsequently, supplementation with three different doses of pomegranate juice also caused non significant decreased of AGE in diabetic rats. The reduced AGE level was not different significantly among groups (Table 2).

There were no significant reduction in total cholesterol and triglyceride levels after treatment with neither

glibenclamide nor pomegranate juice. Besides, HDL cholesterol level was not altered significantly by pomegranate juice.

DISCUSSION

The hypoglycemic effect of pomegranate juice and its effect on malondialdehyde, advanced glycation end product and lipid profiles concentration in STZ-induced diabetic rats were investigated. Pomegranate juice with the three different doses reduced blood glucose level in STZ-induced diabetes rats. The present results were agreed with Khalil (2004) who reported that administration of pomegranate seed extract 0.43 g/kgBW associated with reduction of serum glucose level in alloxan-induced diabetic rats.

Our result indicated that there was a significant reduction in serum concentration of MDA after four weeks treatment with 2 mL/200 g BW of pomegranate juice. The present result was agreed with Tjakradidjaja and Tjakradidjaja (2011), who reported that administration of pomegranate powder 5 and 10% may reduce serum MDA level. According to Turk *et al.* (2008) treatment with 1 ml pomegranate juice within 7 weeks decreased serum MDA level significantly in male rats when compared to the control groups. Study by Atilgan *et al.* (2014) concluded that administration of Pomegranate Juice 0.4 mL/day orally over a period of eight weeks significantly reduced the MDA levels in the serum compared with those in the ischemia group ($p<0.001$). This reduction may associate with the flavonoid antioxidant property of pomegranate that protect tissue from free radical exposure or other pathological process (Shenouda and Vita, 2007).

Our result indicated that there was no significant decrease in serum concentration of AGE in diabetic rats after 4 weeks treatment with three different doses of pomegranate juice. This result did not confirm the previous *in-vitro* study that found significant reduction of AGE level after administration of pomegranate (Dorsey, 2012). Antioxidant property in pomegranate has capability as a free radical scavenger and protects pancreatic beta cells from damage in alloxan diabetic mice given pomegranate (Khalil, 2004).

Serum total cholesterol levels did not show significant reduction after 4 weeks treatment with the three different doses of pomegranate juice. The present results was not agreed with Das and Barman (2012), who reported significant decrease was observed in serum cholesterol after 7 days administration of 500 mg/kg BB/day ethanolic extract of leaves of *Punica granatum* in alloxan-induced diabetic rat. Increased blood insulin level after pomegranate administration may account for this event (Bhaskar and Kumar, 2012). Reduced insulin secretion causes lipolisis with subsequent increases free fatty acid influx into liver and excessive production of VLDL (Goldberg, 2001). Increased VLDL secretion into circulation causes addition cholesterol release into circulation (Murray *et al.*, 2003). In this study, diabetic rats treated with the three different doses of pomegranate juice presented no significant reduction of serum total cholesterol level compared to diabetic rats treated with glibenclamide. Serum HDL and triglyceride levels were not also different significantly after four weeks treatment with pomegranate juice.

Conclusion: Finally, our results demonstrated that pomegranate juice treatment for four weeks significantly reduced blood glucose level, had a tendency to decrease malondialdehyde and AGE serum concentration. It was indicated that pomegranate juice has hypoglycemic and antioxidative effects in STZ-induced diabetic rats.

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