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Phaleria macrocarpa Suppress Nephropathy by Increasing Renal Antioxidant Enzyme Activity in Alloxan-Induced Diabetic Rats

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Abstract – The protective effects of *Phaleria macrocarpa* (PM) against oxidative stress in diabetic rats were investigated. Diabetes was induced in male Sprague Dawley rats using alloxan (150 mg/kg i.p). After the administration of PM fractions for two weeks the diabetic symptoms, nephropathy and renal antioxidant enzymes were evaluated. The results showed that the oral PM treatments reduced blood glucose levels in diabetic rats. The PM fractions decreased kidney hypertrophy and diminished blood urea nitrogen (BUN) in diabetic rats. Malondialdehyde (MDA), a lipid peroxidation marker, was increased in diabetic animals, but was suppressed by the PM treatments. In addition, the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, and glutathione (GSH) level in the alloxan-induced diabetic rats were significantly decreased compared with those in the normal rats, but were restored by PM treatments. The PM fractions also suppressed the level of MDA in the kidney. In conclusion, the anti hyperglycemic and anti-nephropathy of *P. macrocarpa* may be correlated to the increased renal antioxidant enzyme activity in the kidney.
Keywords – *Phaleria macrocarpa*, Hyperglycemia, Diabetic nephropathy, Oxidative stress

Introduction

In 2003 diabetes was the leading cause of end-stage renal disease worldwide. According to WHO, the global prevalence of diabetes for all age-groups has been estimated to increased from 171 million in 2000 to 366 million by 2030 (Wild *et al*., 2004) if no urgent action is taken.

There is growing evidence that the excess generation of highly reactive free radicals largely due to hyperglycemia (West, 2000; Evans *et al.*, 2002), causes oxidative stress which further exacerbates the development and progression of diabetes and its associated complications (Baynes and Thorpe, 1999; Vinik and Vinik, 2003). It is commonly accepted that hyperglycemia-evoked oxidative stress plays a crucial role in the development of diabetic complications (Brownlee, 2001), including nephropathy (Stevens, 2005), which is throughout to result from the augmented generation of reactive oxygen species *via* NADPH oxidase (Gill and Wilcox, 2006). Present diabetes therapy approaches mainly involve drugs that enhance insulin secretion or signaling, as well as inhibitors of endogenous glucose production (Bryla *et al.*, 2003), while the role of antioxidants, which act as important agents for restoring the redox balance of organisms, remains to be established.

Diabetes has been known can be controlled by natural products, therefore, discovery and development of novel drugs for DM is very important. *Phaleria macrocarpa* (Scheff) Boerl (Thymelaeaceae), a medicinal plant originally from Papua, Indonesia has been used in traditional medicine for treating several disease, including diabetes, rheumatism, high blood pressure, and acne, etc (Harmanto, 2003; Winarto, 2003). *P. macrocarpa* (PM) has been reported to contain phenolic glycosides, such as mahkotaside, mangiferin, and kaempferol-3-O-β-d-glucoside, as well as dodecanoic acid, palmitic acid, ethyl stearate, and sucrose (Zhang *et al.*, 2006; Oshimi, *et al*., 2008), and the lignans pinoresinol, lariciresinol, and matairesinol (Saufi *et al.*, 2008). In the in vitro experiments, extracts of PM have been analyzed for their hypoglycemic activities as an inhibitor of enzyme alpha-glucosidase (Sugiwati *et al*., 2006) and anticancer (Triastuti, *et al*., 2006; Faried *et al*., 2007). In a previous study, PM was found to possess anti-diabetic activity in streptozotocin-induced diabetic mice (Triastuti *et al*.,

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2008). In the present study, protection effect of the butanol extract of PM on the generation of free radicals in the kidney caused alloxan was examined

Experimental

Chemicals – Alloxan, thiobarbituric acid, reduced glutathione, 5,5-dithiobis (2-nitrobenzoic acid), Folin°Øs reagent, and bovine albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The bioassay kit for blood urea nitrogen (BUN) was purchased from Asan Company, Korea. All other reagents purchased were of the highest commercial grade available from common commercial suppliers.

Animals – Male Sprague Dawley aged 6 weeks were purchased from Hyochang Science (Daegu, Korea), and maintained under a daily controlled 12 h-light: 12 h-dark lighting cycle at 22 ± 3 °C and 50% humidity on environmentally controlled cabinet with free access to standard food and water *ad libitum*.

Preparation of the *P. macrocarpa* Fractions – Old (red color) *Phaleria macrocarpa* fruits were processed into extract in the Laboratory of Biology and Pharmacy, Department of Pharmacy, Islamic University of Indonesia, Yogyakarta, Indonesia. The pericarps of the fruits were sliced, dried, and ground into powder. To obtain crude methanol extracts, 1 kg of Powder of PM were dissolved 3 times in 2 L of methanol for three days, filtered, and then evaporated. To obtain the ethyl acetate soluble and *n*-butanol soluble parts, half of the methanol extract was then dissolved in 1 L ethyl acetate-water (1 : 1) One liter of water was added to the soluble butanol part, the ethyl acetate, butanol, and water parts were then evaporated. Preparation of the PM fractions yielded methanol (MeOH; 139.54 g), ethyl acetate (EtOAc; 11.07 g, butanol (BuOH; 28.07 g), and water (69.74 g) fractions. All fractions then were lyophilized and used for the experiment.

Induction of Experimental Diabetes – Fasted rat was induced by alloxant (150 mg/kg, i.p) in saline to make Diabetes rats. Normal rats were injected with saline alone. Diabetes was confirmed 72 h after the alloxan injection. Rats with a fasting blood glucose levels above 250 mg/dl were considered diabetic and used in the experiment.

Experimental Groups – Normal and hyperglycemic rats were randomly allocated and similarly grouped into seven groups (five in each): non – diabetic control (normal) group; diabetic control (control) group; diabetic + MeOH fraction 250 mg/kg group; diabetic + EtOAc fraction 250 mg/kg group; diabetic + BuOH fraction 250 mg/kg group; diabetic + water fraction 250 mg/kg group, and diabetic + metformin 150 mg/kg as reference drug. Both normal and control groups were administered an equivalent volume of the vehicle (double distilled water) for the two weeks of the treatment. The PM fractions treatments were started on the fourth day after the alloxan injection and administered orally during the 2 weeks of the treatments. After completion of the treatments, the animals were decapitated after fasting for 18h, with the blood and kidneys collected.

Assay of serum enzymes and components – For the blood glucose analysis, a drop of blood was collected from the tail vein of animals. The blood glucose level was determined using a one touch glucometer (Roche). Serum was extracted from the blood collected directly from the abdominal vein after the rats had been subjected to anesthesia. Serum was separated for the estimation of the blood urea nitrogen (BUN). At the end of the experiment, the kidneys were excised, and then washed thoroughly in ice-cold saline to remove the blood. They were then gently blotted between the folds of a filter paper and weighed. The kidney/body weight ratio was calculated, with the data expressed as an organ index (relative organ weight of one kidney to 100 g of total body weight). Ten percent of homogenate was prepared on 0.1 M phosphate buffer (pH 7.4) using a Teflon homogenizer at 4 °C The homogenate was centrifuged at 3000 g for 20 min to remove the cell debris, unbroken cells, nucleus, erythrocytes, and mitochondria. The cytosolic fraction was used to estimate the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). The Kidney homogenate was analyzed for the reduced glutathione (GSH), lipid peroxidation, and total protein levels.

Assays of renal enzymes – The SOD activity was determined via the ability of the tissue homogenate to scavenge the superoxide anion generated from the photo-illumination of riboflavin according to the method of Marklund and Marklund (1974). The GPx activity was determined by measuring the decrease in the GSH content after incubation of the sample in the presence of H_2O_2 and NaN_3 (Paglia and Valentine, 1967). Renal CAT activity was determined from the rate of decomposition of H_2O_2 (Aebi, 1974). The reduced GSH was determined according to the method of Ellman (1959) and Mitchell *et al.*, (1973), based on the formation of the yellow colored complex with DTNB (5, 5'-dithiobis (2-nitrobenzoic acid). The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1' 1' 3' 3'-tetra-methoxypropane as standard (Ohkawa *et al.*, 1979). The protein

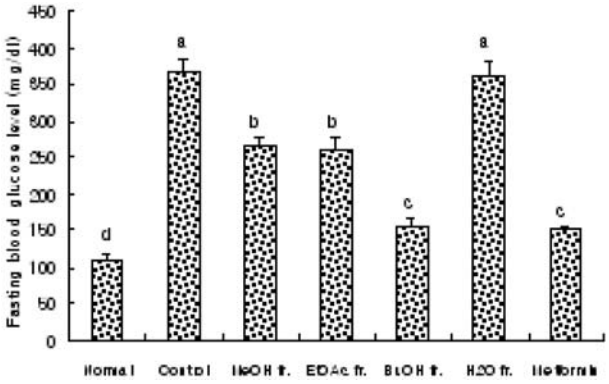

Fig. 1. Effects of the *P. macrocarpa* extracts on the blood glucose levels of alloxan-induced diabetic rats. Values are the means ± SD (n = 6). Values within a barwith different superscripts are significantly different at < 0.05 from the Duncan's test.

content in the tissue was determined (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard.

Statistical Analysis – All statistical analyses were carried out using the SPSS version 12 statistical program (SPSS Inc. Chicago, IL, USA), with data represented as the mean ± SD (n = 6). Significant differences between the mean values were statistically analyzed using one-way analysis of variance, and further analyzed using Duncan's multiple range tests. P values less than 0.05 were considered significant.

Results

In the present study, the administration of alloxan caused diabetic symptoms, such as increased food and water intakes, polyuria, and body weight loss (data are not shown). The blood glucose levels were markedly elevated in the alloxan-induced diabetic rats.

The diabetic rats treated with PM the fractions for the 2 weeks of the treatment showed improvement of diabetic symptoms (Fig. 1). At the end of the treatment, those rats treated with the BuOH fraction showed decreasing of blood glucose levels, but this result was not significantly different from that of metformin (157.4 ± 11.78 and 150.2 ± 7.16 mg/dl respectively); there was no significant difference between the MeOH and EtOAc treated groups (267.4 ± 8.59 and 258.0 ± 17.89 mg/dl respectively). The blood glucose level of the water fractions treated group was not significantly different to that of the control groups (365.0 ± 20.50 and 361.2 ± 17.81 mg/dl respectively) ($p < 0.05$).

At the end of the experimental period, the diabetic rats showed significant renal enlargement compared with the normal group (Table 1). The kidney indices among

Table 1. Relative kidney weight and bood urea nitrogen (BUN) of alloxan-induced diabetic rats

Group	Kidney index	BUN
	g/100 g	mg/dl
Normal	0.9 ± 0.04^{b}	15.8 ± 0.51^{d}
Control	1.4 ± 0.09^{a}	53.5 ± 1.46^{a}
MeOH	1.0 ± 0.02^{b}	28.4 ± 0.59^{b}
EtOAc	0.9 ± 0.03^{b}	29.4 ± 0.71^{b}
BuOH	0.9 ± 0.07^{b}	19.5 ± 0.98^{c}
Water	1.3 ± 0.07^{a}	52.2 ± 1.93^{a}
Metformin	1.0 ± 0.02^{b}	18.0 ± 0.63^{c}

Values are the means ± SD (n = 6). Values within a column with different superscripts are significantly different at $P < 0.05$ from the Duncan's test.

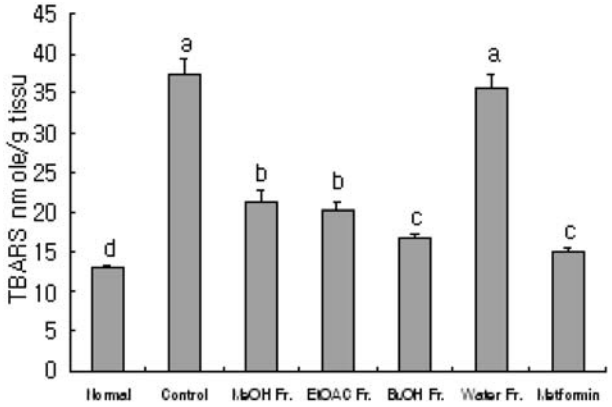

Fig. 2. Effects of the *P. macrocarpa* extracts on the levels of lipid peroxidation in the kidney of alloxan-induced diabetic rats. Values within a bar with different superscripts are significantly different at $p < 0.05$ from the Duncan's test.

the normal, EtOAc, BuOH fractions, and metformin groups were not significantly different to each other but there were differences between these groups and water fraction treated and control diabetic animals. The kidney indices of the normal and control group were 0.9 ± 0.04 and 1.4 ± 0.09 g/100 g, respectively. The elevated blood urea nitrogen in the diabetic rats (Table 1) indicated impaired kidney function. The BUN level in the control animals (53.5 ± 1.46 mg/dl) was increased compared to that in the normal (15.8 ± 0.51 mg/dl) group. The PM fractions, especially the BuOH fraction, decreased the level of BUN in the diabetic rats, but this was not significantly different from the metformin treated group (19.5 ± 0.98 and 18.0 ± 0.63 mg/dl, respectively). The water fraction did not decreased the level of BUN in the diabetic rats, and was not significantly different from that of the control group ($P < 0.05$).

The levels of lipid peroxidation in the kidneys were increased in the diabetic animals (Fig. 2) and treatment

Table 2. Effects of the *P. macrocarpa* extracts on the renal SOD, GPx and CAT activities in alloxan-induced diabetic rats

Group	SOD	GPx	CAT
Normal	18.3 ± 0.67^{a}	2.8 ± 0.17^{a}	1.9 ± 0.43^{a}
Control	2.5 ± 0.69^{d}	1.6 ± 0.16^{d}	0.7 ± 0.09^{d}
MeOH	6.7 ± 0.61^{c}	1.8 ± 0.24^{c}	1.1 ± 0.06^{c}
EtOAc	6.6 ± 1.02^{c}	1.9 ± 0.08^{c}	1.2 ± 0.03^{c}
BuOH	10.9 ± 0.94^{b}	2.2 ± 0.04^{b}	1.5 ± 0.05^{b}
Water	2.4 ± 0.30^{d}	1.6 ± 0.05^{d}	0.8 ± 0.08^{d}
Metformin	11.7 ± 0.85^{b}	2.2 ± 0.07^{b}	1.6 ± 0.26^{b}

Superoxide dismutase (SOD, % inhibitions of NADPH oxidation nmol/min/mg protein), glutathione peroxidase (GPx, nmol NADPH oxidized/min/mg protein), and catalase (CAT, nmol of H_2O_2 consumed/min/mg protein)
Values are the means ± SD (n = 6). Values within a column with different superscripts are significantly different at from the Duncan's multiple range test ($P < 0.05$).

with the PM fractions especially the BuOH fraction, significantly decreased ($p < 0.05$) the lipid peroxidation levels compared to the control group, but not significant difference compared to the metformin treated group. No significant difference ($p > 0,02$) in the level of lipid peroxidation in the kidney was observed between the water fraction treated and control groups.

The activities of renal antioxidant enzymes have been summarized in Table 2. The SOD activity was decreased in diabetic animals but restored by the PM treatment. The PM treatment also showed improving renal GPx and CAT activities. The activity of SOD in the kidney of the control group (2.5 ± 0.69 U/mg protein) was significantly decreased compared to the normal group (18.3 ± 0.67 U/mg protein). Treatment with BuOH fraction increased the SOD activity in the kidneys of diabetic rats. This result statistically not significantly difference compared to the metformin treated group (10.9 ± 0.94 and 11.7 ± 0.85 U/mg respectively), but statistically significant different compare to the control group. The SOD activities in the MeOH and EtOAc fractions treated groups were 6.7 ± 0.61 and 6.6 ± 1.02 U/mg protein, respectively. The water fraction did not increase the SOD activity in diabetic rats and showed no significant differences from that in the control group ($P < 0.05$).

The diabetic rats also showed decreased glutathione peroxidase (GPx) activity compared to the normal group. The GPx activities in the control and normal groups were 1.6 ± 0.25 and 2.8 ± 0.14 U/mg protein, respectively. The BuOH fraction did not significantly increased the GPx activity in the kidney compared to the metformin treated group.

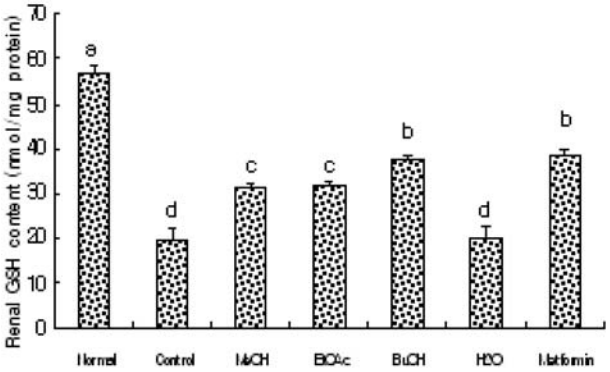

Fig. 3. Effects of the *P. macrocarpa* extracts on the levels of glutathione in the kidney of alloxan-induced diabetic rats. Values are means ± SD (n = 5). Values within a column with different superscripts are significantly different at $P < 0.05$ from the Duncan's test.

The catalase (CAT) activities were decreased in the diabetic animals, but restored by the PM fractions treatments, especially the BuOH fraction. The CAT activity in the control group was 0.7 ± 0.09 U/mg protein, and significantly decreased compared to that in the normal group (1.9 ± 0.43 U/mg protein).

As shown as Fig. 3, the glutathione (GSH) content in the kidneys decreased in diabetic animals. In the control group, GSH content significantly decreased compared to the normal group (19.5 ± 2.66 and 56.8 ± 1.64 U/mg protein, respectively) ($P < 0.05$). The PM treatments especially the butanol fraction, increased the GSH content in rat kidneys.

Discussion

Tight control of blood glucose levels can reduce the complications associated with diabetes, but to optimize recovery and prevent oxidative stress complications alternative treatments are needed (Evans *et al.*, 2002). Glucose can undergo auto oxidation, generate hydroxyl radicals, and react with protein in a non enzymatic manner, which lead to the development of advanced glycation end products (AGEs), with ROS generated at multiple steps during this process (Giardino *et al.*, 1996; Turko *et al.*, 2001); hence, causing diabetic complications, such as neuropathy, retinopathy and nephropathy (Wells *et al.*, 2000). Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodents and many other animal species. This causes insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to diabetes type 1 in humans. Alloxan is selectively toxic to insulin-producing pancreatic

beta cells as it is preferentially accumulated in beta cells through uptake via the GLUT2 glucose transporter. Alloxan, in the presence of intracellular thiols, generates reactive oxygen species (ROS) in a cyclic reaction, with dialuric acid as its reduction product. The toxic action of alloxan towards beta cells is initiated by the free radicals formed in this redox reaction. Alloxan does not cause diabetes in humans (Bansal *et al.*, 1980, Yamamoto *et al.*, 1981). In our study, the activity of the PM against diabetic nephropathy in alloxan-induced diabetic rats was investigated. All rats, that received an alloxan injection developed diabetes. The blood glucose levels in the diabetic animals treated with the MeOH, EtOAc, and BuOH fractions recovered after 14 days of treatments, which was significantly different to that of the control group, but the water fractions treated groups showed no significance difference to the control diabetic group ($P < 0.05$). Since PM fractions can decrease blood glucose levels implies PM can block free radical production and prevent the production of ROS during diabetes. ROS and AGEs are known to have a wide range of chemical, cellular, and tissue effects implicated in the development and progression of diabetic nephropathy including accelerating uremic glomerulopathy with tubulointerstitial damage (Bohlender *et al*., 2005). Diabetic rats showed symptoms of renal nephropathy, such as kidney hypertrophy and elevated serum urea (BUN) (Table 1). After two weeks of treatment, the PM treatment decreased kidney hypertrophy and the BUN value, indicating amelioration in nephropathy. Chronic hyperglycemia was found to produce oxidative stress and increased lipid peroxidation in the kidneys, as shown by the increased level of renal malondialdehyde as a lipid peroxidation level marker (Ohkawa *et al*., 1979). Lipid peroxidation in diabetes mellitus increased by increased oxidative stress in the cell as a result of persistent hyperglycemia, which depletes the antioxidant scavenger systems (Mahboob *et al*., 2005; Akkus *et al*., 1996). This effect of hyperglycemia was again ameliorated by the PM treatment; in line with a previous report, which showed that PM decreased lipid peroxidation, possibly due to an antioxidant mechanism (Triastuti *et al.*, 2008). In our study, the antioxidant enzymes activities in kidney were analyzed to confirm the antioxidant activity of PM in diabetic rats. The MDA levels in the PM treated animals were significantly decreased compare to the control diabetic group, which was correlated with the elevated level of antioxidant enzyme in the kidney.

SOD, CAT and GPx are enzymes that destroy peroxides and play significant roles in providing antioxidant defenses to organisms (Robertson *et al*., 2003). Diabetic rats showed reduced SOD, CAT, and GPx concentrations. The PM treatments, especially the butanol fraction, increased the activity of these enzymes and; thus, may help to avoid the generation of free radicals during diabetes mellitus.

GSH, a major non protein thiol plays an important role in coordinating the body's antioxidant defense processes. The results in this study indicate that alloxan-induction lowered the levels of GSH in the kidney. Improvement of renal GSH levels in diabetic rats by the PM treatment confirm its anti-oxidative effect

Abnormally high levels of lipid peroxidation, with the simultaneous decline of antioxidant defense mechanisms, can cause damage to cellular organelles and lead to oxidative stress. In our study, the antioxidant enzymes activities were decreased; hence, the lipid peroxidation levels in the diabetic animals were increased. The MDA levels were significantly decreased in the PM treated animals compare to the control diabetic group, which correlated with the elevated level of antioxidant enzymes in the kidney.

In conclusion, the results of the present study indicate that orally administered *P. macrocarpa* treatment protects against alloxan-induced nephropathy in rats by enhancing the renal antioxidant activity and decreasing lipid peroxidation.

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