Piper Sarmentosum Influences The Oxidative Stress Involved In Experimental Diabetic Rats

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Citation

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Abstract

Introduction:
Piper sarmentosum (P.s) is an herbal plant that is widely used as food and traditional medicine and it is known to possess antioxidant as well as glucose lowering actions. Diabetes Mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia. DM complications are linked to increased oxidative stress and impaired antioxidant mechanisms. Malondialdehyde (MDA), an oxidative stress biomarker and superoxide dismutase (SOD), an antioxidant enzyme are linked to oxidative stress. Objective:
The present study aimed to observe the effects of P.s aqueous extract on malondialdehyde levels and superoxide dismutase activities of diabetic rats. Methods:
Diabetes was induced in 30 male rats with streptozotocin (50 mg/kg, intramuscular; dissolved in 0.9% NaCl solution). A group of eight normal rats (I) were given vehicle in the similar volume (intramuscular; 0.9% NaCl solution). The diabetic rats were categorized into three groups; control diabetes (II), insulin treated diabetes (5 IU/kg Insulatard HM, subcutaneous daily) (III) and P.s treated diabetes (0.125 g/kg, per oral daily) (IV). Following 28 days, blood samples were obtained to examine the levels of serum MDA and erythrocyte SOD activities. Results:
P.s treated diabetes rats (group IV) showed a significant decrease ($p<.05$) in MDA level (2.5 µM) and a significant increase ($p<.05$) in erythrocyte SOD activity (8.1 U/mgHb) compared to control diabetes rats (group II). Conclusion:
Aqueous extract of P.s exhibits ameliorative effects on the oxidative stress in diabetic animals and helps in checking the complications arising out of DM.

INTRODUCTION

Diabetes Mellitus (DM) has gradually becoming a global health problem. World Health Organization (WHO) estimated that about 171 million people worldwide were affected with DM in year 2000. It is projected that the disease will have 366 million sufferers by year 2030.[1] In Malaysia, the National and Health Morbidity Survey (NHMS) has reported the prevalence of DM in people above 30 years to increase from 6.3% (NHMS I) to 14.9% (NHMS III) between year 1986 and 2006. It is estimated that Malaysia is expected to have a total of 2.48 million people with DM by 2030.[2] About 4 million deaths occurring due to DM were account to approximately 6.8% of deaths, worldwide.[3]

Hyperglycaemia is observed in DM together with the derangement of carbohydrate, fat and protein metabolism in the body. The increase in glucose level is attributed to reduction in insulin secretion or action or both, accompanied by reduced usage of glucose and increased gluconeogenesis.[4] DM is extremely linked to increased oxidative stress resulting from both increased formation of free radicals and impaired mechanism of the antioxidant defense system.[5] Glucose auto-oxidation occurs secondary to hyperglycaemia, resulting in production of free radicals such as reactive ketoaldehyde and superoxide anion radicals.[6] Increased oxidative stress is a recognised factor for both the progression of diabetes and development of its complications.[5] Chronic hyperglycaemia in DM is associated with long term complications involving the vital organs such as kidneys, heart, eyes, nerves and blood vessels.

Oxidative stress affects biomolecules such as lipids and proteins leading to lipid peroxidation. Malondialdehyde (MDA), one of the by products of lipid peroxidation, is a useful marker for oxidative stress damage in the body.

Superoxide dismutase (SOD) is a group of metalloenzymes, which help in catalyzing dismutation of superoxide anion radicals to oxygen and hydrogen peroxide. This has an important role in cellular antioxidant defense mechanism.[8] To date, three types of SOD were identified. The enzyme is characterized by the types of metal, which are Cu-Zn SOD, Mn SOD and Fe SOD. SOD can be found in high concentration in organs like the brain, heart, liver, kidney and erythrocyte.[8]

The last few decades have witnessed progressive and dramatic evolution of therapeutic approach of DM in the direction of reduction of morbidity and symptoms, normalization of glucose level, prevention of diabetic complications and prolongation of disease free survival. The current trend tends to favour the use of some medicinal plants such as Tinospora crispa, Gymnema sylvestre, Eugenia jambolana and Piper sarmentosum as alternative treatment to oral hypoglycemic agents and insulin.[9] This can reduce incidence of adverse effects and usage of expensive medicines. P.s has been identified to possess high antioxidant property[10] as well as glucose lowering properties.[11,12]

Piper sarmentosum Roxburgh (L. piper = pepper and sarmentosum = twiggy, with long slender runners) belongs to the family of Piperaceae. It is a creepy terrestrial herb about 20 cm tall that grows wild in the forest and commonly found in the Asian countries such as Malaysia, Cambodia, Myanmar, Thailand and Philippines. Closely resembling the betel leaf plant, it is characterized by simple, alternate and chordate leaves with lancelet-elliptical blades.[13] For more than two decades, considerable research has focused on the effects of extracts of P.s as anti-bacterial, anti-tuberculosis, anti-inflammatory, anti-nociceptive, anti-carcinogenic and many others.[14-17] Aqueous extract of P.s at an oral dose of 0.125g/kg was reported to lower blood glucose for 7 days.[11] Methanol extracts of P.s at an oral dose of 0.25g/kg was found to reduce blood glucose only for the first 3 weeks of the study.[12] Apart from that, P.s possesses natural superoxide scavenger, Naringenin, which contributes to the high antioxidant property.[10] To date, there has been little study focusing on the effects of PS on the oxidative stress in diabetic rats.

In the light of the literature presented, the present study was planned to investigate the effect of P.s aqueous extract on the serum levels of MDA and erythrocyte SOD activity in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

PLANT

Fresh leaves, stems, fruit and flowers (5kg) of P.s were obtained from local market. Authentication of the whole plant was done by a botanist and a voucher specimen (UKMB 29849) was deposited in the Herbarium Unit, Department of Science Biology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

PREPARATION OF P.S EXTRACT

All extraction procedures were performed at the Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia and Forest Research Institute of Malaysia (FRIM). The plants were cleaned with tap water and dried at room temperature for one week before been chopped into small pieces. The chopped plant was finely grinded using a mechanical blender and stored at room temperature prior to extraction. The powdered plant was suspended in distilled water (1:10) at 70^{o}C for 24 hours and then concentrated using heat.[11] The collected extract was pooled and filtered twice with Whatman 2 filter paper. The filtrate was combined and freeze-dried in FRIM to form powder. The powdered extract was stored at 4^{o}C until further use.

ANIMALS

This study was approved with ethics clearance granted by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). Animal care and handling was in accordance with the institutional guidelines for animal research of Universiti Kebangsaan Malaysia. Healthy adult male Sprague Dawley rats weighing between 150-200g were obtained from the Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia. Each rat was placed in a polycarbonate cage in an air-conditioned animal house at temperature of 24^{o}C and illuminated for 12 hours daily (lights on from 0700 to 1900) throughout the experiment. They were fed with standard rat chow (Gold Coin, Malaysia) and tap water ad libitum.

EXPERIMENTAL DESIGN AND INDUCTION OF DIABETES

The animals were randomly categorised into four main groups. Thirty rats were induced diabetes while another eight rats were taken as group I (normal control). DM was induced in overnight fasted rats by a single intramuscular injection of Streptozotocin (STZ, Sigma Chemical Co., USA) at a dose of 50 mg/kg body weight, dissolved in cold normal saline (0.9% NaCl solution, B.Braun Malaysia)

within 30 minutes of preparation. DM was confirmed 72 hours later by determining the fasting blood glucose (FBG) using a glucometer and glucose reagent strip (Accu-Chek Advantage, Roche, USA). The STZ-treated rats were considered to be diabetic if their FBG levels were > 8 mmol/L.[18] Group I received a single dose of normal saline via intramuscular injection at a similar volume. All rats were left alone for another 10 days before commencing treatment.

The diabetic induced rats were further categorised into three groups. Group II (control diabetes) (n=10) received normal saline only by oral gavage daily. Group III (diabetes treated with insulin) (n=10) received subcutaneous injection of Insulatard HM at 5 IU/kg body weight daily.[19] Group IV (diabetes treated with PS) (n=10) received P.s water extracts at 0.125 g/kg body weight by oral gavage daily.[11] Group I (n=8) received normal saline only by oral gavage daily. All treatments were given at the same time at 0900 hours throughout the experiment period.

Following 28 days of treatment, the rats were anaesthetized via inhalation of diethyl ether. Venous blood was obtained by puncturing the retro-orbital sinus with heparinized tubes. Blood was collected directly into plain and EDTA tubes in a cold box. Blood in plain tubes were allowed to clot. On the same day, all blood samples from both plain and EDTA tubes were centrifuged at 3000 rpm at 4°C for 10 minutes.

DETERMINATION OF MDA LEVEL

Sera from centrifuged plain tubes were collected for determination of the MDA level. The levels were estimated using a commercial MDA kit (Northwest Life Science Specialties). The principle of the assay allows the MDA to react with thiobarbituric acid (TBA) to form a coloured complex. The absorbance was read at 532 nm using a spectrophotometer and the final unit was taken as µM.

DETERMINATION OF SOD ACTIVITY IN ERYTHROCYTE

Plasma and white buffy layers from centrifuged EDTA tubes were carefully removed. The erythrocyte precipitates were washed and resuspend in normal saline at three times the volume and centrifuged at 3000 rpm at 4°C for 10 minutes. This step was repeated three times. The washed cells were lysed in deionised water at 4 times the volume and mixed thoroughly. The mixture was centrifuged at 10,000 x g at 4°C for 15 mins.[20] The haemoglobin in the haemolysates were estimated using Cyanmethemoglobin method (Eagle Diagnostics, USA). The haemolysates were then used for determination of the erythrocyte SOD activity.

The activity of erythrocyte SOD activity was estimated using the Superoxide Dismutase Assay Kit (Cayman, USA). The assay utilizes tetrazolium salt for detection of the superoxide radicals generated by xanthine oxidase and hypoxanthine. A unit of SOD is the amount of enzyme needed to cause 50% dismutation of the superoxide radicals. The absorbance was read at 450 nm using a plate reader. The activity of erythrocyte SOD was determined by dividing the measured SOD in haemolysate with its haemoglobin concentration and the final unit is taken as U/mgHb.

STATISTICAL ANALYSIS

Data were expressed as mean and standard deviation (SD). They were analyzed statistically with non-parametric tests, Kruskal-Wallis and Mann-Whitney for comparisons between groups using the Statistical Package for Social Sciences version 16 (SPSS Inc., USA). Differences were considered significant at $p<0.05$.[21]

RESULTS

SERUM MDA LEVEL

Group IV had the lowest mean level of MDA. Group II showed the highest mean level of MDA, followed by the Group III and Group I were shown in Table 1. However, no significant differences were seen between the four groups. Nevertheless, there was significant reduction of MDA level ($p<0.05$, Mann-Whitney) in Group IV compared to Group II (Figure 1).

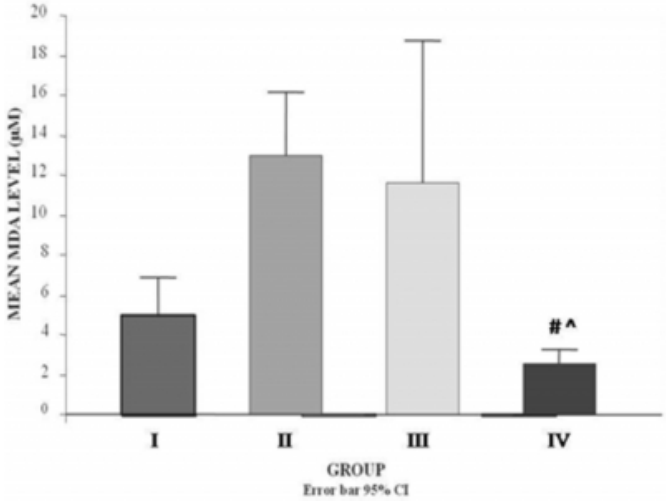
Figure 1

Table 1 Mean serum level of malondialdehyde (MDA) between groups after 28 days of treatment (n=24)

Group	Mean (*sd*) (µM)
I (n=6)	4.9 (2.14)
II (n=6)	13.0 (3.40)
III (n=6)	11.6 (6.95)
IV (n=6)	2.5 (0.92)

Figure 2

Figure 1 Bar chart showing the mean serum level of malondialdehyde (MDA) after 28 days of treatment



ERYTHROCYTE SOD ACTIVITY

Group IV showed the highest mean activity of erythrocyte SOD. The lowest was in Group II, followed by Group I and Group III (Table 2). However, no significant differences were seen between the four groups. Nevertheless, there was significant increase of erythrocyte SOD activity ($p<0.05$, Mann-Whitney) in Group IV compared to Group II (Figure 2).

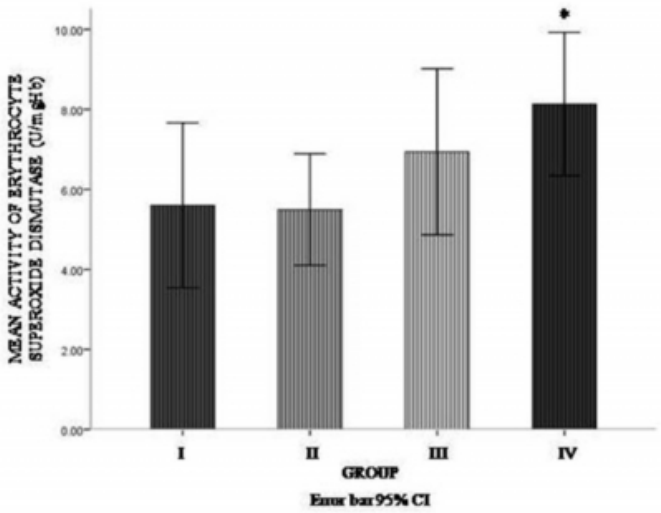
Figure 3

Table 2 Mean activity of erythrocyte superoxide dismutase enzyme between groups after 28 days of treatment (n=31)

Group	Mean (*sd*) (U/mgHb)
I (n=8)	5.6 (3.54)
II (n=7)	5.5 (4.10)
III (n=9)	6.9 (4.87)
IV (n=7)	8.1 (6.35)

Figure 4

Figure 2 Bar chart showing the mean activity of erythrocyte superoxide dismutase (SOD) after 28 days of treatment



DISCUSSION

Oxidative stress, a characteristic feature of DM, has been associated with the long term diabetic complications.[22-23] The causative mechanisms contributing to oxidative stress in DM include enzymatic, non-enzymatic and mitochondrial causes. Glucose oxidation is the non-enzymatic mechanism in oxidative stress. Hyperglycaemia causes glucose oxidation and glycosylation of protein resulting in increased production of free radicals such as hydroxyl radical, oxygen singlet, superoxide and hydrogen peroxide that leads to DNA damage.[24]

Membrane phospholipids are the major target for free radicals oxidative damage resulting in a condition known as lipid peroxidation. The latter is a chain of reactions involving radical complex resulting in the production of by-products such as dienes conjugate, lipid hydroperoxide and malondialdehyde. Malondialdehyde (MDA) level is a biomarker for oxidative stress.[25] Apart from overproduction of free radicals, diabetic complications are linked to accumulation of lipid peroxidation by-products.[26]

Nitric oxide synthase, NAD(P)H oxidase and xanthine oxidase are the enzymes involved in the oxidative stress in DM. The NAD(P)H oxidase enzyme mediates the formation and increased the superoxide radicals in this disease.[27] Formation of superoxide radicals at the level of mitochondria is the first step in initiating the oxidative stress cycle in DM. The mitochondrial radical oxygen species is also linked to the pathogenesis of complications of DM.[28] Superoxide dismutase is one of the key enzymes responsible

for radical scavenging activities. It catalyzes superoxide radicals to hydrogen peroxide and oxygen. Then, hydrogen peroxide is detoxified into water by the action of catalase or glutathione peroxidase in mitochondria. In DM, there are impaired antioxidant defense mechanisms, leading to worsening of the oxidative stress.[29]

Apart from these, other mechanisms contributing to the oxidative damage in DM include the changes in energy metabolism, changes in levels of inflammatory mediators and the impairment of antioxidant defense systems.[30] The overall increase in free radicals and impaired antioxidant mechanism results in oxidative stress in DM. This in turn leads to the damage in cells and tissues in the body, with sequel in acute and chronic complications in DM.[31]

In the present study, lipid peroxidation was measured using serum levels of MDA via thiobarbithuric acid reaction (TBAR). The increase in serum TBAR indicated enhanced lipid peroxidation leading to release of its byproduct (MDA) into circulation. It also indicated some pathological changes such as tissue injury and failure of the antioxidant defense mechanism to scavenge the excess free radicals.[32] The SOD enzyme was specifically studied as it is the first antioxidant defense system in erythrocyte involved in haemoglobin auto-oxidative reaction.[29]

Our study showed that streptozotocin (STZ) increased the level of serum MDA and lowered the activity of erythrocyte SOD as revealed by the control diabetes rats. However, it was not significantly different from the control group. This finding is consistent with that of Crouch et al.(1978) who reported a 30-40% reduction of SOD activity in the diabetic rats following 5 days induction with STZ compared to the normal rats.[33] Nishikagi et al. (1981) reported the MDA levels increased in diabetic patients as compared to normal subject.[34] Streptozotocin produces its cytotoxic effect via glutathione oxidation. It was observed that STZ was formed of glucosamine glucopyranose ring, which binds to glucosamine nitrogen and nitrosurea group. The glucose molecule binds to glucose receptor on the -cells in the Islets of Langerhans of pancreas, while the nitrosurea causes the cell damage. At the same time, there is reduction in the reduced glutathione level in erythrocytes.[33]

Other studies have reported different levels of SOD activities on diabetic conditions. The Cu-Zn SOD in erythrocyte was low in type 2 DM.[35] Those with type 1 DM were found to have high erythrocyte SOD. It is noteworthy that the erythrocyte SOD activity may decrease, increase or have no significant difference.[36-38] The reduction in the SOD activity is possibly due to a decrease in antioxidant defense or large amount of free radicals overcoming the defense system. High free radicals augment oxidation process which inhibits the SOD enzyme.[39] However, increase SOD activity at the early stage of type 1 DM is considered a compensatory mechanism arising from the increase in superoxide radicals.[40] This may explain the insignificant low SOD activity in the group II diabetic rats as they were induced diabetes for only 6 weeks before blood was taken.

This study also revealed that insulin treatment administered to the group III diabetic rats was able to increase the erythrocyte SOD activity more than the group I, even though the difference was not significant. This finding compares favourably with the observations of Wiryana, where insulin treatment given to critical patients with diabetes had increased their erythrocyte SOD activity.[41] Zbronska et al. (1995) had documented that insulin treatment was able to enhance erythrocyte SOD of patients and delays the diabetic complications.[42] Surprisingly, the serum MDA level was higher in insulin treated diabetes rats (Group III) compared to control (Group I) and PS treated diabetes rats (Group IV). This obviously indicates that PS is more potent at reducing oxidative damage than the gold standard insulin treatment. A study on male Sprague Dawley rats receiving insulin therapy with normal blood glucose level did not show an increment in oxidative stress level.[43] However, diabetic rats with poor glycaemic control treated with insulin did show increased in oxidative stress level and reduction in antioxidant capacity in the liver.[43] This probably explains the elevated levels of MDA in insulin treated diabetes rats in this study, which were found to have high glucose levels despite given insulin treatment. Maintaining normal blood glucose level is the key to minimize oxidative stress.

Piper sarmentosum has long been known to possess high antioxidant property and radical scavenging activity. The methanolic extract of PS was reported to have high superoxide scavenging assay (88%) compared to the standard SOD.[10] A natural antioxidant compound, Naringenin (4',5,7-Trihydroxyflavanone) with 75.7% superoxide scavenging activity, has been identified in PS. Naringenin, a highly potent natural antioxidant that belongs to the flavanoid group, has been reported in the raw materials of larch and grapefruit extracts.[10] Animal experimentation showed that flavanoid reduced high level oxidative stress damage revealed by the decreased in serum TBAR levels of liver and kidney in diabetic-induced rats.[44]

Fruits and vegetables have been reported to posses antioxidant properties which can combat degenerative diseases in which free radical damage are involved in the pathophysiology.[45] The protective action of antioxidants causes inhibition of free radical-induced chain reactions preventing structural deterioration of lipids in membranous organelles.[46] Hence, antioxidant treatment is beneficial in diseases associated with oxidative stress.

In this study, treatment with PS water extract significantly decreased the serum MDA level and increased the erythrocyte SOD activity in the diabetic rats compared to control diabetes rats. This finding supports the existing evidence of the highly effective antioxidant action by P.s. By increasing the cell's defense mechanism system, damage to cells and tissues can be reduced and occurrence of complications in DM can be delayed. Moreover, no significant difference was seen when Group IV was compared to Group III. This clearly indicates that P.s aqueous extract reduces the oxidative damage and improves the antioxidant defense mechanism in the diabetic rats comparable to the effect of insulin.

CONCLUSION

The link between DM and oxidative stress has thrown light on novel therapeutic approach. In the quest for new therapeutic strategies that can reduce oxidative stress, our findings suggest the effective use of P.s as an antioxidant to reduce the diabetic complications. In conclusion, P.s aqueous extract reduced oxidative stress as well as increased the antioxidant defense mechanism in DM. Future studies are advocated to corroborate the findings with respect to appropriate dosage and side effects if any.

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