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Full Length Research Paper

Antioxidant effect of methanol-derived extract from *Dorstenia picta* (Moraceae) twigs in streptozotocin-induced diabetic rats

Ngueguim Tsofack Florence[a*], Dzeufiet Djomeni Paul Desire[a], Donfack Jean Hubert[b], Ngo Lemba Esther[a], Tsala Emery David[a], Renaud Beauwens[c], Kamtchouing Pierre[a] and Dimo Théophile[a*].

[a]Department of Animal Biology and Physiology, Laboratory of Animal Physiology, University of Yaounde I, P. O. Box 812 – Cameroon.
[b]Department of Biochemistry, Laboratory of Pharmacology and Toxicology, University of Yaounde I, P.O. Box 812 - Cameroon.
[c]Laboratory of Cellular and Molecular Physiology, Université Libre de Bruxelles, P.O. Box 1070 – Belgium.

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This study was undertaken to determine the *in vivo* and *in vitro* antioxidant effect of methanol-derived extract from *Dorstenia picta* twigs. Diabetes was induced by streptozotocin injection in male wistar rats. After two weeks, treatment with either *Dorstenia picta* plant extract or insulin was initiated. Antioxidant activities of the plant extract were investigated by evaluating the levels of malondialdehyde, reduced glutathione and some antioxidant enzymes such as superoxide dismutase and catalase. In addition β-carotene linoleic acid model system was used for the evaluation of the *in-vitro* antioxidant activity and the total phenolics content of the plant extract was also estimated. Daily administration of methanol-derived extract from *D. picta* during 14 days significantly decreased the blood glucose levels. The treatment indicates the reduction of lipid peroxidation and the improvement of antioxidant parameters. We found that, in comparison with untreated diabetic control rats, oral administration of methanol-derived extract from *D. picta* at the dose of 150 mg/kg induced a significant decrease in lipid peroxidation, catalase and superoxide dismutase activities with an increase in glutathione level. *In-vitro* study showed that, the extract contains 16.77 GAE/µg of total phenolics and possess antioxidant activity with IC_{50} value 32.26 ±1.27 µg/mL in β-carotene bleaching inhibition assay. These results indicate that methanol-derived extract from *D. picta* twigs controls glycaemia, improves the oxidative status thus decrease the damage caused by oxidative stress in diabetic rats.

Keywords: *Dorstenia picta*; chronic hyperglycaemia; oxidative stress; streptozotocin

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by abnormalities in carbohydrate, lipid and protein metabolism which not only lead to hyperglycemia but also cause many complications such as retinopathy, nephropathy, neuropathy and hypertension. Hyperglycemia observed in diabetics is a widely known cause of free radicals production which induces tissue damage. Hyperglycaemia induces increase in glucose uptake and the rise in glucose concentration leads to activation of intracellular pathways including aldose reductase, protein kinase C, nicotinamide adenine dinucleotide phosphate oxidase, mitochondrial electron-transport chain (Couture, 2004). Activation of these pathways lead to over production of reactive oxygen species and intracellular formation of advanced glycation end products (AGEs) with associated toxic effects. In addition to these direct intracellular effects, glucose and particularly fructose and sorbitol react non-enzymatically

*Corresponding author Email: tsngueguim@yahoo.fr ;or dimo59@yahoo.com Phone: (237) 99642510

with proteins, lipids, and nucleic acids to produce AGEs in the extracellular medium, which in turn induce reactive oxygen species generation (Singh, 2001; Couture, 2004). Oxidative damage occurs as a consequence of an imbalance between the formation and inactivation of oxygen free radicals. This process leads to the destruction of membrane lipids and production of lipoperoxides and their products. Inactivation and removal of Reactive oxygen species (ROS) depend on relations with antioxidative defense mechanisms (Lipinski, 2001; Serafini and Del Rio, 2004). Oxidative stress has been considered to be a common pathogenic factor of diabetic complications and thus appears a target for therapeutic treatments. Inhibition of oxidative stress blocks the manifestations of the disease (Ha and Kim, 1999). In diabetes, antioxidant defences are blunted whereas the generating systems of reactive oxygen species are stimulated (Dickinson et al., 2002; Hodgkinson et al., 2003). There is an increase interest over the last few years in the protective action of some medicinal plants in the prevention of free radical mediated diseases.

Dorstenia is one of the genus in the large moraceae family that produces small herbaceous plants. *D. picta* is commonly used in traditional Cameroonian medicine to treat many diseases like hypertension and diabetes mellitus. Other traditional uses of Dorstenia species are against headaches and abdomenal pains (Raponda-Walter and Sillans, 1969), snakebites, infections and rheumatism (Abegaz et al., 2000). We have previously shown that, aqueous extract and methanol-derived extract of *D. picta* possess antidiabetic properties in streptozotocin-induced diabetic rats (Ngueguim et al., 2006; Ngueguim et al., 2007).

The main objective of this study was to investigate antioxidant properties of methanol-derived extract from *D. picta* twigs on streptozotocin-induced diabetic rats.

MATERIAL AND METHODS

Chemical

All chemicals and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

Extraction of plant materials

Twigs of *D. picta* were collected from Ngoumou in the Central Province of Cameroon in August, 2005. They were identified by Dr. Zapfack Louis of the Department of Plant Biology and Physiology, Faculty of Science, University of Yaounde I. The voucher specimen (No. 57063) was deposited at the National Herbarium, Yaounde, Cameroon. The air-dried twigs of *D. picta* were powdered and 2 kg macerated in 2 L of methanol for 48 h at room temperature. Removal of the solvent from the extract under reduced pressure yielded 200 g (10 %) of a dark green residue. The residue was mixed with hexane to remove its hydro-insoluble component. Following filtration, the filtrate was dissolved in ethyl acetate. The final residue obtained (neither soluble in hexane nor ethyl acetate) constituted the methanol residue extract of *D. picta* (60 g). The purpose of this extraction procedure was to obtain a hydro-soluble organic extract which is close to aqueous extract normally used traditionally. This extract was solubilised in distilled water prior to administration to the experimental animals.

Animals

Male Wistar albino rats weighing 200-250 g, raised in the Faculty of Science, University of Yaounde I, were used. They were kept and maintained under standard laboratory conditions of temperature, humidity, 12-hours light-dark natural cycle and allowed access to rat chow and water *ad libitum*. Fasting rats were deprived of food for at least 12 h but not water. Fasted rats with blood glucose levels of 93.96 ± 2.34 mg/dL were used in our experiments. The studies were conducted according to the guidelines of the Cameroon National ethical Committee on the use of laboratory animals for scientific research (Ref n° FW-IRB00001954).

Induction of experimental diabetes in rats

Diabetes mellitus was induced by intravenous (penile vein) injection of 55 mg/kg of streptozotocin in 0.9% sterile sodium chloride solution to non-fasted rats anesthetized with ether. Control group received normal saline through the same route. Four days later, blood glucose levels of streptozotocin treated fasted rats greater than 300 mg/dL were considered as diabetic and used in this study. Rats were kept for 14 days before the beginning of the treatment to stabilize the diabetic condition and to allow a permanent and chronic hyperglycaemia (Jyoti et al., 2002).

Treatment

Forty rats were divided into five groups made of 8 rats each: group I made of normal rats received by oral administration 10 mL/kg of distilled water (served as vehicle). Groups II, III, IV and V (diabetic rats) were treated as follow: group II served as diabetic control received also the vehicle, group III was administered insulin subcutaneously (10 UI/kg), groups IV and V were treated by gastric intubation with methanol-derived extract from *D. picta* twigs respectively at the doses of 75 and 150 mg/kg.

Fourteen days after injection of streptozotocin, plant extract or insulin were given daily during two weeks. Blood glucose levels were determined weekly using glucose oxidase method (Accuchek glucometer, Boehringer Mannheim, Germany) (Dzeufiet et al., 2006; Ngueguim et al., 2007). At the fifteenth days of treatment, rats were sacrificed under ether anaesthesia. The liver, kidney and aorta were removed immediately and used for preparation of homogenate (20%). The supernatant was kept at -20°C for analysis of antioxidant parameters such as Malondialdehyde (MDA), reduced glutathione, catalase and superoxide dismutase.

In vivo antioxidant activity assay

Assay of malondialdehyde (MDA)

The method of Wilbur et al. (1949) was used for MDA determination. Briefly, 250 µL of distilled water and 20 µL of homogenate were mixed in a test tube. 250 µL of Tris-HCl buffer (pH 7.4), 500 µL of TCA (20%) and 1000 µL of TBA (0.67%) were added after which the mixture was heated at 90°C for 10 min in a water-bath. After cooling at room temperature, the tubes were

Table1: Effect of methanol derived-extract from *D. picta* twigs on blood glucose levels in streptozotocin- induced diabetic rats.

Treatment	Blood glucose (mg/dL)			
	D 0	Day 7	Day 14	Variation (%)
Normal control	85.25 ± 02.32	87.25 ± 02.28	87.25 ± 01.60	02.35
Diabetic control	434.75 ± 23.05	405.25 ± 05.94	458.25 ± 33.83	05.40
Insulin (10 UI/kg)	455.66 ± 15.64	156.66 ± 20.27**	109.33 ± 07.83**	76.00
D. picta (75 mg/kg)	438.35 ± 12.67	276.15 ± 38.28*	227.15 ± 14.73*	48.20
D. picta (150 mg/kg)	424.00 ± 18.56	203.5 ± 85.84*	99.00 ± 4.04**	76.65

Values are mean ± SEM, n = 8, * P<0.05, ** P<0.01 compared with diabetic control.

centrifuged at 3000 rpm for 15 min. The absorbance of the pink coloured supernatant was measured at 530 nm. The MDA concentration was calculated using an extinction coefficient of $1.56.10^5$ cm^2/mM. Lipid peroxide was expressed in term of Pmol/g of tissue.

Reduced glutathione level (GSH)

Reduced glutathione level was determined according to the method of Ellman (1959). 3 mL of Ellman reagent was added to a test tube containing 200 µL of tris-HCl buffer (50 mM, pH 7.4) and 200 µL supernatant. The solution was mixed and kept at room temperature for 1 hour. The absorbance was measured at 412 nm. The GSH concentration was calculated using an extinction coefficient of 13600 $M.cm^{-1}$ The Reduced glutathione level was expressed in term of nmol/g organ.

Assay of superoxide dismutase activity (SOD)

The method of Misra and Fridovich (1972) was used. This method involves the inhibition of epinephrine autooxidation in an alkaline medium. In the mixture containing 134 µL of sample and 1666µL of carbonate buffer (pH, 10.2), 200 µL of epinephrine of sample were added. The solution was rapidly mixed and the absorbance was read at 20 seconds and 80 seconds at 480 nm in UV spectrophotometer. The activity was expressed as a percentage inhibition of autoxidation in the tested sample, and this percentage was then expressed as units/mg protein.

Assay of catalase activity

Catalase activity in liver, kidney and aorta tissue was determined according to the method followed by Beers and Sizer (1952). Briefly 250 µL of sample was added to a tube containing 250 µL of 0.1M phosphate buffer at pH 7.2. The reaction was started by addition, of 1000 µL of 30 mM H_2O_2. The absorbance of the sample was read at 240 nm at 30 seconds intervals for a total of 90 seconds against blank. Catalase activity was calculated using Beer-Lamberts law according to Audigie et al. (1993) and was expressed as UI/mg protein.

***In vitro* antioxidant assay**

β-carotene bleaching inhibition assay

The antioxidant activity of plant extract was evaluated using β-carotene linoleic acid model system (Kabouche et al., 2007). β-carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of tween-80 emulsified mixture. Chloroform was evaporated at 40°C using a rotary evaporator. Thus, 100 mL of distilled water saturated with oxygen was slowly added to the residue and the solution was vigorously agitated to form a stable emulsion. 4 mL of this mixture was added into the test tubes containing 200 µL of plant extract prepared in methanol at final concentrations (25 ,50, 100, 200 and 400 µg/mL). As soon as the emusified solution was added to the tubes, zero time absorbance was measured at 470 nm. The tubes were incubated for 2 hours at 50°C. Vitamin C was used as standard. Antioxydant activity was calculated as percentage of inhibition (I%) relative to the control using the following equation:

$$I\% = [1-(A_s-A_{s120})/A_c-A_{c120})]$$

A_{so} was initial absorbance, A_{s120} was the absorbance of the sample at 120 min, A_c was initial absorbance of negative control and A_{c120} was the absorbance of the negative control at 120 min. The test was done in three replicates.

Determination of plant total phenols

The plant total phenols were determined using Folin-Ciocalteu reagent (Chang et al., 2001). 0.02 µL of 100 µg/mL of plant extract was mixed with 1.58 mL of water followed by the addition of 100 µL of Folin-Ciocalteu reagent. The mixture was kept at room temperature (25 ± 2°C) for 8 minutes followed by the addition of 300 µL of 25% Na_2CO_3. After 2 hours of incubation at room temperature, the absorbance was measured at 765 nm against the blank. The total phenol content was determined using the following equation that was obtained from the standard curve of gallic acid (dissolved in methanol):

Absorbance= 0.0024x - 0.001 (R^2=0.988)

Statistical analysis

Results are expressed as the mean ± SEM. Statistical differences between control and treated groups were tested by one way analysis of variance (ANOVA) followed by Dunnett-s test using graphpad Instad version 3.06 Software. P values less than 0.05 were considered to be significant.

RESULTS

Effect of plant extract on blood glucose level

Before the treatment with plant extract, the fasting blood glucose concentration of streptozotocin-induced diabetic rats were more than 5 times higher than those of the non-diabetic rats (Table 1). Diabetic rats treated with plant

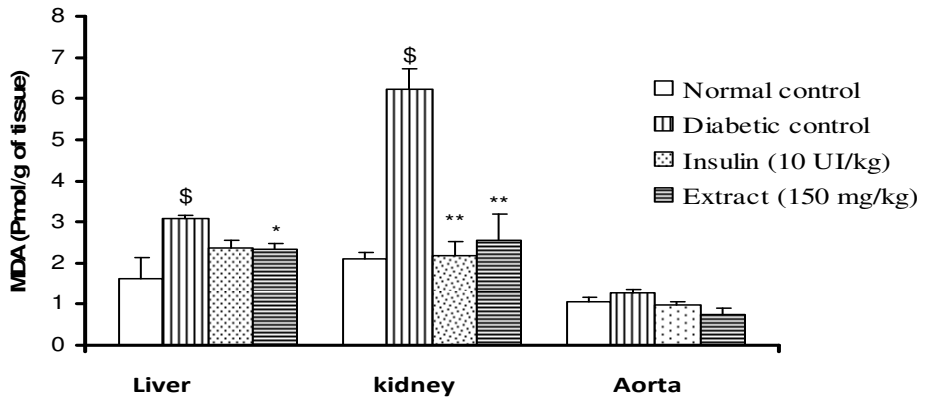

Figure. 1. Effect of methanol derived-extract from *D. picta* twigs on malondialdehyde in streptozotocin-induced diabetic rats.
Values are mean ± SEM, n = 8, * P<0.05, ** P<0.01 compared with diabetic control, $ P<0.05, compared with normal control.

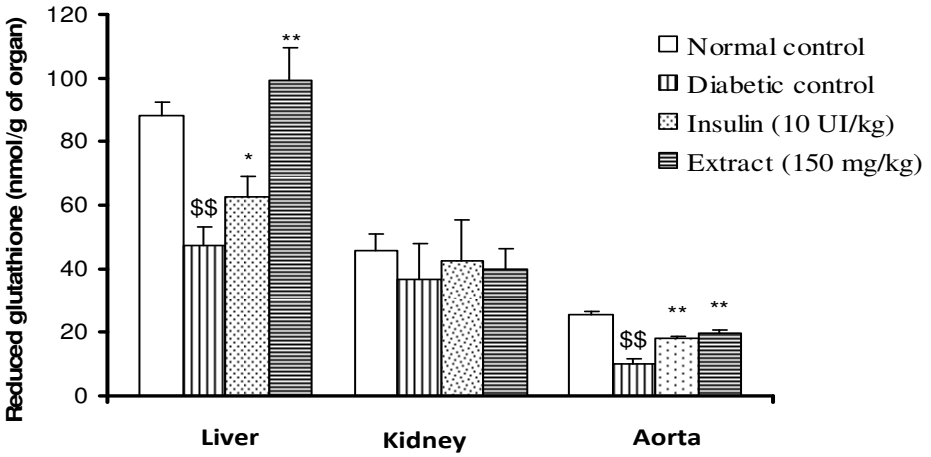

Figure 2. Effect of methanol derived-extract from *D. picta* twigs on reduced glutathione levels on streptozotocin-induced diabetic rats.
Values are mean ± SEM, n = 8, * P<0.05, ** P<0.01 compared with diabetic control, $$ P<0.01, compared with normal control.

extract showed a significant and dose dependant decrease in blood glucose after 14 days of repeated oral treatment. This decrease was 50.43 % and 78.39% for 75 and 150 mg/kg, respectively compared to the diabetic control group. It is important to mention that the plant extract at all doses significantly reduced (P<0.05) the blood glucose levels after one week of treatment. The plant extract at the dose of 150 mg/kg was more effective than 75 mg/Kg and was used to evaluate *in-vivo* antioxidant parameters.

Effect on antioxidant parameters

Figure 1 shows the effects of treatment on lipid peroxidation (MDA). Lipid peroxidation in liver and kidneys was significantly increased by 47.24% and 65.91% respectively in diabetic control group as compared to normal group. In aorta, there was no significant increase in lipid peroxidation in diabetic rats treated with plant extract (150 mg/kg) as compared with diabetic control. Treatment with plant extract at the dose of 150 mg/kg exhibited a significant decrease in the extent of lipid peroxidation of 25.24% and 58.68% in liver and kidney respectively. Diabetic control rats were also characterized by a significant decrease in reduced gluthathion concentration by 46.60% and 39.08 respectively in liver and aorta as compared to normal control (Figure 2). Fourteen consecutive days plant administration at the dose of 150 mg/kg induced a significant increase of reduced glutathione concentration by 52.59 % and 55.89% respectively in liver and aorta. No significant changes were observed in kidney.

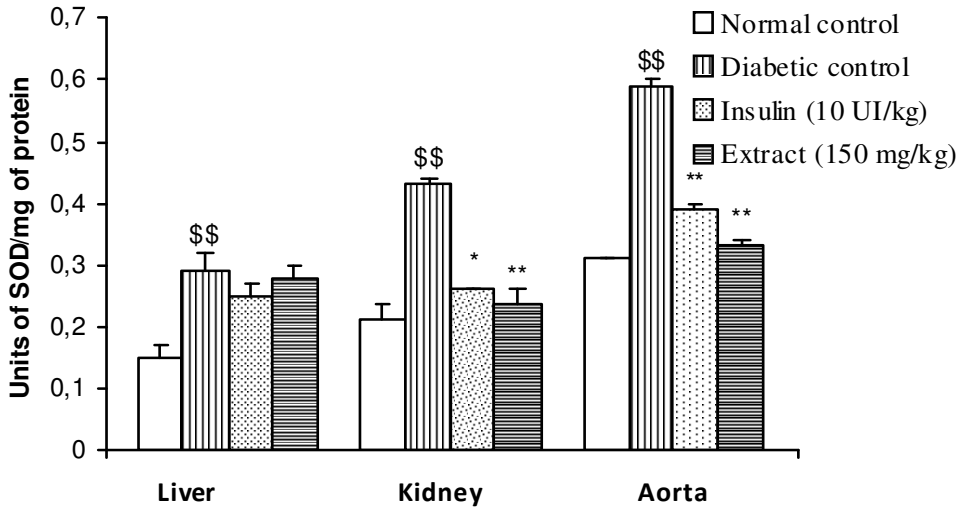

Figure 3. Effect of methanol derived extract from *D. picta* twigs on superoxide dismutase on streptozotocin-induced diabetic rats.
Values are mean ± SEM, n = 8, * P<0.05, ** P<0.01 compared with diabetic control, $^{\$\$}$ P<0.01, compared with normal control.

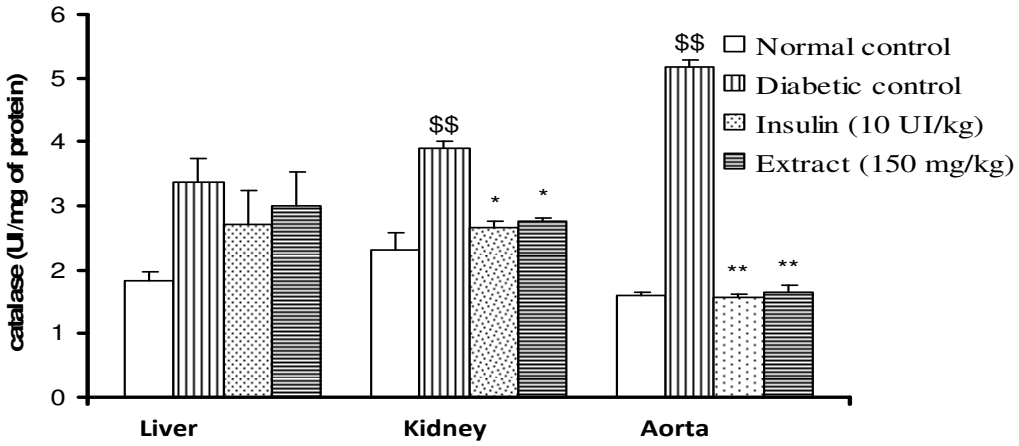

Figure 4. Effect of methanol derived-extract from *D. picta* twigs on catalase on streptozotocin-induced diabetic rats.
Values are mean ± SEM, n = 8, * P<0.05, ** P<0.01 compared with diabetic control, $^{\$\$}$ P<0.01, compared with normal control

In diabetic group, the activity of superoxide dismutase (SOD) was significantly increased by 48.27%, 51.11% and 47.50% respectively in liver, kidney and aorta (Figure 3). On the other hand, the catalase activity was also significantly increased by 45.23%, 48.18% and 69.17% in liver, kidney and aorta respectively when compared to the normal group (Figure 4). *D. picta* significantly (P<0.01) improved the activities of SOD and catalase by 44.06% and 66.30% respectively in aorta as compared to the diabetic group. At the level of the kidney, the reduction was 44.18% and 29.15% respectively for SOD and catalase activities. The improvement of these two parameters was not significant in liver and kidney after plant administration.

β-carotene bleaching inhibition assay

The effects of various concentrations of plant extract (25 to 200 µg/mL) against β-carotene peroxidation are shown in Figure 5. Compared to vitamin C, the plant extract at the dose of 50 to 200 µg/mL showed a significant lower activity against lipid peroxidation. The inhibition percentage of plant extract (25, 50, 100, and 200 µg/mL)

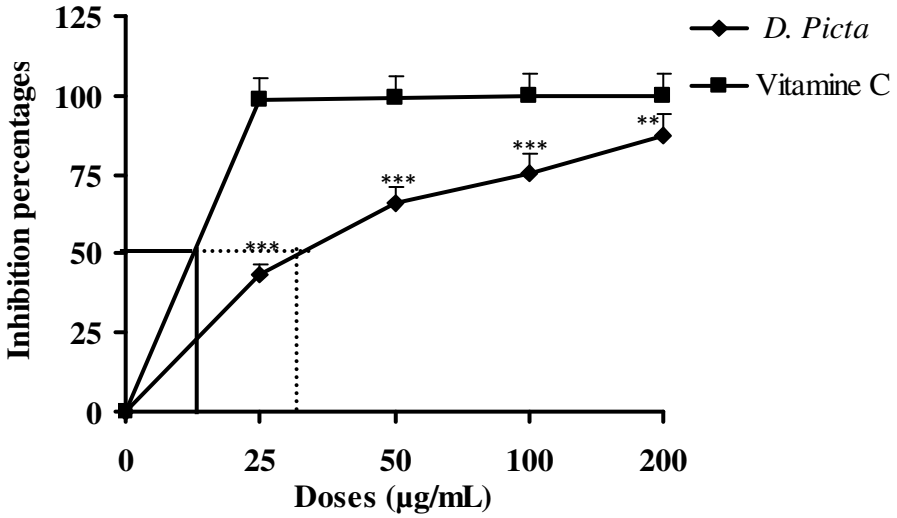

Figure 5. Effect of methanol derived-extract from *D. picta* twigs on the oxidative degradation of β-carotene. Values are means ± SEM, n = 3. ** P<0.01, *** P<0.001 compared with vitamin C.

in linoleic model acid system was dose dependant and varying from 43.16 ± 0.72 to 87.16 ± 0.57%. At the same concentration, the inhibition percentages of vitamin C was 60.15, 98.17 ± 1.2, 99.38 1.28, 100.00 ± 0.00 and 100.00± 0.00%, respectively. These inhibition percentages were used for the determination of the IC_{50} values, which were 32.26 ±1.27 µg/mL and 15.26±1.20 µg/mL for plant extract and vitamin C respectively.

In vitro antioxidant activity assay

The extract was found to contain 16.77 gallic acid equivalent /µg of extract total phenolics.

DISCUSSION

In a previous study, we showed that methanol-derived extract of *D. picta* twigs possess an antidiabetic activity (Ngueguim et al., 2007). Since hyperglycemia has been observed in diabetic induced reactive oxygen species (ROS) which induced-tissues damage by autooxidation of glucose, increase glycolysis, activation of sorbitol pathway and non-enzymatic protein glycation, we extended our investigation to the *in-vivo* and *in-vitro* antioxidant effect of this plant extract. In diabetic control group, elevated fasting blood glucose levels from initial to final confirm and uncontrolled diabetes (animal with a permanent hyperglycemia) whereas *D. picta* extract remarkably reduced blood glucose concentration in diabetic rats. The plant extract lowered blood glucose level gradually and raised the normal value (99 mg/dL) at the end of treatment at the dose of 150 mg/kg. This effect was comparable to synthetic insulin which also exhibited hypoglycaemic effect. In diabetes, the generating systems of reactive oxygen species are stimulated (Dickinson et al., 2002; Hodgkinson et al., 2003).

Glucose autoxidation and protein glycation can generate free radicals that catalyze the lipid peroxidation (Altan et al., 2006) which in turn contribute to increase oxidative stress and the damage to antioxidant defence system. Oxidative stress is associated with the peroxidation of cellular lipids, which could be determined by the measurement of lipid peroxidation products among which is MDA (malondialdehyde) (Lipinski, 2001). Therefore, the concentration of lipid peroxidation products may reflect the degree of oxidative stress in diabetes. It has been previously reported that, the level of MDA is increased in tissues and blood of Streptozotocin-induced diabetic rats (Kakkar et al., 1995; Baynes and Thorpe, 1999; Aylin et al., 2007). Furthermore, the increased level of lipid peroxidation products results in increased levels of oxygen free radicals, which attack the polyunsaturated fatty acids in cell membranes and cause lipid peroxidation (Kehrer, 2000). In our study we investigated the direct effect of the plant extract on lipid peroxidation by measuring MDA level. In diabetes group, MDA was found to be higher than those of normal animals, indicating lipid peroxidation. The MDA level in the liver and kidney of diabetic rats administered *D. picta* extract was significantly decreased as compared to the diabetic control rats. This result suggests that the plant extract could improve the pathological condition of diabetics by reduction of lipid peroxidation. This finding was confirmed by *in-vitro* tests where the plant extract was found to induce inhibition of β-carotene oxidation. The effect of plant extract was also studied in the

enzymatic antioxidant defences. Superoxide dismutase (SOD) and catalase activities were significantly increased in diabetic rats. This confirms the reaction of diabetic rats against oxidative stress resulting from hyperglycemia. The same observation was reported by Ahmed (2005) who showed the physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defence system. There are several studies with varying results in enzymatic antioxidant defences in diabetes. Piper et al. (1995) demonstrated that, in experimental diabetes, catalase activity increase in vascular tissues. This is in contrast to Aylin et al. (2007) who reported a decrease in SOD and catalase activities of liver tissue in alloxan induced-diabetes. In addition, Wohaieb and Godin (1987) showed increased activities of catalase and superoxide dismutase in the pancreas of diabetic rats while the liver showed a decrease in the activities of catalase and SOD. The treatment with the plant extract during 14 days significantly reduced SOD and catalase activities. On other hand, the level of reduced glutathione was significantly reduced in diabetic rats, suggesting the inactivation caused by reactive oxygen species and ongoing treatment with plant extract induced the increased of reduced level of glutathione. It has been reported that in diabetic rat, there is a decrease in the concentration of reduced glutathione mainly due to the decrease in activities of enzymes involved in GSH synthesis, in the transport rate of GSSG (Murakami et al., 1989; Ahmed, 2005) and a decrease in the activity of glutathione reductase which acts to reduce GSSH to GSH (Tagami et al., 1992; Ahmed, 2005). It might be possible that the active principles would be interacting in different pathways such as increase in the regeneration of oxidized glutathione (GSSG) or increase the activity of glutathione reductase which reduce GSSG to GSH.

Preliminary phytochemicals analysis of the plant extract showed the presence of polyphenols, flavonoids and triterpenes (Ngueguim et al., 2007) which are known to possess antioxidant activity (Montilla et al., 2003; Hennebelle et al., 2004). In the present study, the extract was found to contain 16.77 gallic acid equivalent /µg of extract total phenolics .Thus, the antioxidant activity could be attributed to the presence of phenolic compounds presents in the plant extract.

CONCLUSION

Methanol-derived extract from *D. picta* twigs administered orally to streptozotocin-induced diabetic rats reduce oxidative stress by controlling the serum glucose level, improving the levels of some antioxidant compounds and enzymes thereby playing an important role in the prevention of the pathogenesis of diabetic complications. Further investigations on isolation of the active compounds responsible for the antioxidant activity are required.

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