Free Radicals Scavenging Potential Of The Aqueous Extract Of Viscum Album (Mistletoe) Leaves In Diabetic Wistar Rats Hepatocytes

H Nwanjo

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Abstract

The aqueous extract of Viscum album leaf was investigated for its possible hypoglycaemic and antioxidant potentials in streptozotocin (STZ) induced diabetic Wistar rats. A single I.P. injection of STZ at the dose of 65 mg/kg body weight elevated the glucose levels > 205 mg/dl after 3 days. Treatment with the aqueous extract at the dose of 100 mg/kg and 200 mg/kg body weight resulted in significant reduction ($P<0.05$) in blood glucose levels. Body weights were significantly reduced ($P<0.05$) in STZ-Induced diabetic rats when compared to normal rats while the extract significantly ($P<0.05$) prevented a decrease in body weight in the V. album treated diabetic rats. The aqueous extract of the V. album leaf administration also resulted in decreased levels of malondialdehyde (MDA) and increased levels of reduced glutathione (GSH), vitamins C and E and the activities of superoxide dismutase (SOD) and Catalase (CAT) thus resulting in reducing the free radical formation in blood and liver tissues of the diabetic rats. These observations demonstrated that aqueous extract of Viscum album leaf have strong hypoglycaemic effect and in vivo antioxidant activity in STZ-Induced diabetic rats and was dose dependent.

INTRODUCTION

Diabetic mellitus and other numerous pathological events such as atherosclerosis and inflammation processes are associated with the generation of relative oxygen species (ROS), and consequently the induction of several chain reactions among them, lipid peroxidation and others (Cross et al, 1987). Accumulating evidence suggests that oxidative cellular injury caused by free radicals contributes to the development of diabetes mellitus (Bambolkar and Sainani, 1995). Free radicals are either generated by cellular metabolisms such as glycolysis, mitochonchrial respiration, and xenobiotic detoxification or by exogenous factors such as red-ox reactions. Some are extremely reactive and therefore interact with some “vital” macromolecules including lipids, nucleic acids and proteins (Nia et al, 2003). The cells have numerous defense systems (enzymic and non-enzymic antioxidants) to counteract the deleterious effects of ROS and free radicals. Moreover, diabetes also induces changes in the tissue content and the activity of the antioxidant enzymes (Genet et al, 2002).

Recently, some synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been suspected to be dangerous to human health (Safer et al, 1999). Therefore there is an urgent need to search for novel antioxidants from natural sources, which could be used in medicine and additives to nutriceuticals (Thomas and Wade, 2001).

Viscum album commonly knows as mistletoe and awushie in Igbo language belongs to family loranthaceae, is a unique semi parasitic plant growing on various deciduous host trees and shrubs (Kernzehok et al, 1997). The part of the plant mainly used for medicinal purposes is the leaf and twig part. Viscum album has been claimed to be antidiabetic (Obatomi et al, 1994), immunomodulatory (Solar et al, 1998), bacteriostatic (Fulder, 1998), antihypertensive and reduces cholesterol level (Nkanu et al, 2002) and therapeutic values for many other ailments. The herb has been analyzed to contain lectins, viscotoxin, polysaccharides and alkaloids as active constituents (Leoper, 1999).

The effectiveness of the plant to remedy ailment conditions in trado-medical practices may not be unconnected with the natural products present in the plant and their abilities to act as radical scavengers. For this reasons it was therefore reasonable to investigate the free radical scavenging activities of Viscum album in diabetic Wistar rats.

MATERIALS AND METHODS

CHEMICAL

Streptozotocin (STZ), Thiobarbituric acid (TBA), butylated hydroxytoluene (BNT), reduced glutathione (GSH), 2,2,-dipyridyl, xynol orange, aspartic acid, alanine, -ketoglutarate, 2,4 – dinitro phenyl hydrazine (DNPH), 5,5 – dithiobis-2- nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co (St Louis, MO-USA). The rest of the chemical utilized were of analytical grade and were obtained from local firm (Nigeria).

PLANT MATERIALS

Mistletoe (Viscum album) leaves used for this study were obtained from kola nut tree (host plant) from Anara in Isiala Mbano Local government Area of Imo State. Dr S.E Okeke, Department of Plant Biology and Biotechnology, Imo State University, Owerri confirmed the botanical identification of the plant leaf. The voucher samples are kept in the university herbarium for reference.

PREPARATION OF THE AQUEOUS PLANT EXTRACT

The method for leaf process was carried out according to ($_{8}$). Fresh leaves of Viscum album collected from the host plant were first washed free of sand and debris. Grinding was done using Thomas Contact Mill (Pye Unicam, Cambridge, England). A quantity o the ground sample was soxhlet extracted using 500ml distilled water for 12 hour at 100°C. The extract was evaporated to dryness using Grant instrument, Cambridge, England) at 45°C -50°C to a yield of 20g plant material which was dissolved with appropriate volume of water.

PHYTOCHEMICAL STUDIES

The chemical classes of constituent in the freshly prepared extract were detected using standard photochemical reagents and procedures as described by Tease and Evens ($_{20}$).

In general, test for the presence or absence of phytochemical compound using the above methods involve the addition of an appropriate chemical agent to the crude material in a test tube. The mixture is then shaken vigorously or gently as the case may be. The presence or absence of saponins, flavonoids, tannins, alkaloids etc. was observed.

ANIMALS

Male Wistar rats (200-300g) bred in the Animal House of College of Medicine and Health Sciences, Imo State University, Owerri were used in this study. They were housed in stainless steel cages and kept in a room where a 12-hour light/dark cycle was maintained. They were allowed free access to water and feed diet (product of Pfizer Nigeria Ltd) throughout the period of the experiment.

INDUCTION OF DIABETES IN RATS

After one week of acclimatization, the rats were subjected to a 16 hour fast. Diabetes was induces with a single intraperitoneal injection of streptozotocin (STZ) at a dose of 65mg/kg body weight. The STZ was freshly dissolved in citrate buffer (0.01m, pH 4.5) (Ozsoy, Sacan, 2000). The injection volume was prepared to contain 1.0 ml/kg (Murali et al, 2002). After 5 days, blood glucose levels were measure and the animals with a concentration of more than 230 mg/dl were classified as diabetic (Cetto et al, 2000).

EXPERIMENTAL DESIGN

Twenty four male Wistar rats were used in this study. The rats were randomized and divided into four groups of six animals each.

Group 1: Normal, received normal saline solution (0.9% NaCl w/v, 5 ml/kg).

Group 2: Diabetic, received STZ (65 mg/kg body weight) once

Group 3. Diabetic, receive STZ (65 mg/kg body weight) once before receiving aqueous extract of Viscum album (100 mg/kg body weight)

Group IV: Diabetic received STZ (65 mg/kg body weight) once before receiving aqueous extract of Viscum album (200 mg/kg body weight).

After 14 days of treatment, all the rats were decapitated after fasting for 16 hours. Blood was collected in two different tubes i.e. one with fluoride oxalate anticoagulant for plasma separation and another without anticoagulant to separate serum for various biochemical estimations. The livers were dissected out and cleared off blood. They were immediately transferred to ice-cold containers containing 0.9% Nacl and homogenized in 0.1N Tris-Hcl buffer (pH 7.4), and used for the estimation of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) activity.

MEASUREMENT OF BLOOD GLUCOSE LEVELS

The body weight and blood glucose levels were measured at

the beginning and end of the experiment. Blood samples were obtained by tail vein puncture of both the normal and STZ-induced diabetic rats. Blood glucose levels were determined using a glucometre (Lifescan Johnson and Johnson Company, Milipitas, CA).

ACUTE TOXICITY TESTS

The acute toxicity of the extract was tested using 30 Wistar rats divided into 5 groups of 6 rats each, with each group receiving graded dose (200-1000 mg/kg body weight, intraperitoneally) of the aqueous extract of Viscum album as described by Ghosh, (1984). After administration of the extract the rats were observed for toxic effects after 48 hours treatment. The toxicological effects were observed in terms of mortality expressed as LD_{50}. The number of animals dying during a period was noted. The LD_{50} of the extract was estimated from the graph of percentage (%) mortality (converted to probit) against log-dose of the extract, probit 5 being 50% (Litch field, et al, 1959).

ESTIMATION OF SERUM LIPIDS

Extraction of lipids from serum carried out according to the procedure of Folch et al, (1957) by using chloroform-methanol (2:1 v/v) mixture. From this total cholesterol LDL- and HDL – cholesterol triacylglyceroles and phospholipids were estimated as described earlier by us (Nwanjo, 2005; Nwanjo and Ojiako, 2005,).

ESTIMATION OF LIPID PEROXIDATION

Lipid peroxidation in plasma and liver was estimated colorimetrically by measuring malondialdehyde (MDA) by the method of Nichans and Samualson (1968) in brief, 0.1ml of plasma was treated with 2ml of (1:1:1 ratio) TBA – TCA – HCL reagent (TBA 0.37%: 0.25N HCL: 15% TCA) and placed in water bath for 15min, cooled and centrifuged and then clear supernatant was measured at 535 nm against reference blank.

ESTIMATION OF ENZYMIC ANTIOXIDANTS

Superoxide dismutase (SOD) activity was determined by the modified method of NADH-phenazinemethosulphate-nitrobluetetrazolium formazon inhibition reaction spectrophotometrically at 560 nm (Kakkar et al, 1984). A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction /min/mg protein.

Catalase (CAT) was assayed calorimetrically as described by Sinha (1972) using dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1.3 ratio). The intensity was measured at 620nm and the amount of hydrogen peroxide hydrolyzed was calculated for the catalase activity.

ESTIMATION OF NON-ENZYMIC ANTIOXIDANTS

Reduced glutathione (GSH) was determined by the method of Ellman (1959). 1ml of supernatant (0.5ml plasma/0.5ml liver homogenate precipitated by 2ml of 5% TCA) was taken and 0.5ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 3ml of phosphate buffer (pH 8.0) were added. The colour developed was read at 412nm.

Vitamin C (ascorbic acid) concentration was measured by Omaye et al, (1979) method. To 0.5ml of plasma/0.5ml liver homogenate, 1.5ml of supernatant, 0.5ml of DNPH reagent (2% DNPH) and 4% thiourea in 9N sulphuric acid) was added and incubated for 3 hours at room temperature. After incubation 2.5ml of 8.5% sulphuric acid was added and colour developed was read at 530nm after 30 min.

Vitamin E (-tocopherol) was estimated by the method of Desai (1984). Vitamin E was extracted from plasma/liver homogenate by addition of 1.6ml ethanol and 2.0ml petroleum ether to 5.0ml plasma and centrifuged. The supernatant was separated and evaporated. To the residue, 0.2ml of 0.2% 2, 2, - dipyridyl, 0.2ml of 0.5% ferric chloride was added and kept in dark for 5min, an intense red colour layer obtained on addition of 4ml butanol was read at 520nm.

STATISTICS

Statistical evaluation of data was performed by using one-way analysis of variance ANOVA followed by Duncan's multiple range test (DMRT) (Duncan 1957).

RESULTS

EFFECT OF EXTRACT OF VISCUM ALBUM ON BLOOD GLUCOSE LEVELS AND BODY WEIGHT CHANGES

The results of blood glucose levels and body weight changes in normal, STZ-induced diabetic rats and Viscum album treated diabetic rats were shown in Table 1. There was a significant ($p< 0.01$) increase in blood glucose levels in STZ-induced diabetic rats (Group II) when compared with normal rats. Administration of aqueous extract of Viscum album at a dose of 100 and 200 mg/kg body weight significantly ($p<0.05$) decreased blood glucose in STZ induced rats (Group III and IV). The results were found to be in a dose dependent manner.

The body weight changes in diabetic group was significantly decrease ($p<0.05$) when compared with the normal control which then returned to near normal in diabetic rats treated with aqueous extract of Viscum album at a dose of 100 and 200 mg/kg body weight

EFFECT OF AQUEOUS EXTRACT OF VISCUM ALBUM ON SERUM LIPIDS

The changes in the levels of serum lipids in control and experimental rats are illustrated in Table 2. The total-cholesterol, LDL-Cholesterol and triacylglycerol significantly increased and HDL-Cholesterol and phospholipids significantly decreased in STZ-induced diabetic rats (group II) ($P< 0.05$) when compared with the normal (group I) rats. The aqueous extract of Viscum album (100 mg/kg and 200 mg/kg body weight) offered a significant protection against alteration in the serum lipids of diabetic rats. The results were also dose dependent.

EFFECTS OF VISCUM ALBUM AQUEOUS EXTRACT ON MDA AND SOME ENZYMIC AND NON-ENZYMIC ANTIOXIDANT STATUS OF CONTROL AND EXPERIMENTAL RATS

The levels of MDA in plasma and liver were significantly ($p<0.05$) increased in STZ-induced rats as compared to normal rats. Treatment with aqueous extract of Viscum album resulted in a significantly decrease in the levels of lipid peroxidation products (MDA) in diabetic rats.

EFFECTS OF VISCUM ALBUM AQUEOUS EXTRACT ON ENZYMIC ANTIOXIDANT STATUS OF CONTROL AND EXPERIMENTAL RATS

A significant decrease ($P< 0.05$) in the activities of enzymic antioxidants such as superoxide dismutase (SOD) and catalase (CAT) of the liver was noted in STZ-induced diabetic rats when compared with the normal rats. Upon administration of 100 and 200mg/kg body weight of aqueous extract of Viscum album, the activities of both SOD and CAT were significantly reversed to near normal.

EFFECTS OF VISCUM ALBUM AQUEOUS EXTRACT ON NON-ENZYMIC ANTIOXIDANT STATUS OF CONTROL AND EXPERIMENTAL RATS

The levels of plasma and liver vitamins C, E and reduced glutathione (GSH) were significantly depleted in STZ – induced diabetic rats. Treatment with aqueous extract of Viscum album significantly increased the levels of these non-enzymic antioxidants in diabetic rats.

DISCUSSIONS

The aqueous extract of Viscum album (mistletoe) leaves was assayed to evaluate their potential as scavenger of tree radicals which are the origin of peroxidative stress found to be fundamental in the pathogenesis of several conditions such as diabetes mellitus, hypertension, atherosclerosis, AIDS, and skin problems such as acceleration of skin ageing (Bonina et al, 1998; Clostre, 1999). Streptozotocin (STZ) causes a significant elevation in the level of blood glucose in rats. Administrations of 100 and 200 mg/kg body weight of aqueous extract of Viscum album leaves significantly decreased the blood glucose level in these rats suggesting that it has an hypoglycaemic properties. The change in body weight of the rats given the plant extract shows the aqueous extract of V. album has a significant effect of controlling the loss of body weight which is caused during diabetes and was dose dependent.

Membrane lipids succumb easily to deleterious actions of (reactive oxygen species) ROS (Reiter, 1995). STZ is toxic to pancreatic -cells and is thus widely used for induction of experimental diabetes mellitus in animals, resulting in the production of ROS (Mazunder et al, 2005).

In the present study the increased level of malondialdehyde (MDA) levels in plasma and liver of rats treated with STZ reflected the lipid peroxidation as the consequence of oxidative stress caused by STZ. Oxidative stress is associated with the peroxidation of cellular lipids, which is determined by measurement of TBA- reactive substances. The concentration of lipid peroxidation products may reflect the degree of oxidative stress in diabetes. It has been reported previously (Raynes, 1991, Kakkar et al, 1995 Mazunder et al, 2005) that tissues and blood of rats with STZ-induced diabetes, malondialdehyde, the product of lipid peroxidation is increased. Moreso, the increased level of MDA results in increased levels of oxygen free radicals, which attack polyunsaturated fatty acids in cell membranes and cause lipid peroxidation. STZ can also give rise to oxygen free radicals because of the increased blood glucose level in diabetes (Rakieten et al, 1963).

Administration with aqueous extract of Viscum album leaf protects the cells through attenuation of lipid peroxidation and decreased the production of free radical derivatives, as evident form the decreased levels of plasma and liver MDA levels. Thus the plant extract offers protection against oxidative stress (khopde et al, 2000) by scavenging of free radicals.

Reduced level of non-enzymic antioxidants such as reduced glutathione in (GSH), vitamin C and vitamin E on STZ administration were observed in this study. Administration of aqueous extract of the Viscum album leaf to STZ-induced diabetic rats, maintained the level of non-enzymic antioxidants to near normal, by the possible role of the herb improving the GSH status. GSH in a major endogenous antioxidant which counterbalance free radical mediated damage and it is well known that GSH is involved in the protection of normal cells structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reactions

It is well established that GSH in blood keeps up the cellular levels of the active forms of vitamins C and E by neutralizing the free radicals. When there is a reduction in the GSH the cellular levels of vitamin C is also lowered, indicating that GSH, vitamin C, and vitamin E are closely interlinked to each other (Paris and Amali 2005).

The enzymic antioxidant defense systems are the nature protector against lipid peroxidation. Superoxide dismutase (SOD) scavenges the superoxide ions produced as cellular by products. SOD is a major defense for aerobic cells in combating the toxic effects of superoxide radicals (McCrod et al, 1976). Catalase (CAT) reduces hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals thereby protecting the cellular constituents from oxidation damage in peroxisomes. The decreased activities of SOD and CAT in STZ-induced diabetic rats result in the accumulation of superoxide radicals and H2O2 respectively. These enzymes prevent generation of hydroxyl radices and protect the cellular constituents from oxidative damage (Pari and Amali, 2005). The increased activities of SOD and CAT in Viscum album administered diabetic rats may result from scavenging of radicals generated by diabetic induced lipid peroxidation thereby decreasing the utilization of these antioxidant enzymes to reduce the diabetic induced oxidative treat. This might be responsible for the increased activities of antioxidant enzymes on administration of this plant extract.

This investigation shows that the aqueous extract of V. album leaves in addition to being hypoglycaemic seems to be effective for reducing oxidative stress and free radical-related diseases including diabetes.

Figure 1

Table 1: Mean values of body weight and blood glucose in experimental and control rats

	Body weight (g)			Fastng blood glucose (FBG) (mg/dl)	
Treatment	Initial	Final	change	Initial	Final
Control (Group 1)	260.68 ± 9.2	276.88 ± 8.4	16.2 ± 3.6**	89.64 ± 8.4	86.7 ± 7.9
Diabetic Group II	272.4 ± 6.2	258.71± 6.9	-13.69 ± 2.4	216.38 ± 25.3	230.7 ± 27.5
Diabetic + 100 mg/kg *V. album* (Group III)	263.7 ± 10.4	272.20 ± 5.2	8.5 ± 4.2*	211.95 ± 13.7	158.53± 10.8#
Diabetic + 200 mg/kg *V. album* (Group IV)	267.6 ± 13.2	280.42 ± 12.8	12.82±2.8**	234.23 ± 14.3	128.78 ± 5.9#

*Significantly different from diabetic rats (group II) (P<0.05).

**Significantly different from II and Group III (P<0.05).

Significantly different from initial FBG (P<0.05).

Figure 2

Table 2: Mean values of serum lipids in experimental and control rats.

Treatment	Total-C (mg/dl)	HDL-C (mg/dl)	LDL-C mg/dl	TG (mg/dl)	Phospholipid (mg/dl)
Control (Group 1)	106.3 ± 5.8**	45.8± 2.7*	45.76 ± 2.5**	73.7 ± 2.8**	102.2 ± 4.3*
Diabetic (Group II)	181.7 ± 6.9	28.9 ± 5.3	123.28 ± 3.6	147.6 ± 4.4	61.8 ± 2.8
Diabetic + 100 mg/kg *V. album* (Group III)	143.8 ±7.4*	40.2 ± 5.0*	81.08 ± 2.9*	112.6 ± 6.2*	84.3 ± 4.7*
Diabetic + 200 mg/kg *V. album* (Group IV)	97.6 ± 5.5**	36.5 ± 4.6*	45.22 ± 3.0**	79.4 ± 5.6**	80.2 ± 6.2*

*Significantly different from diabetic rats (Group II) (P<0.05)

**Significantly different from group II and Group III (P<0.05)

Figure 3

Table 3: Mean values of plasma and liver lipid peroxidation in experimental and control rats.

Treatment	Plasma MDA (nmol/ml)	Liver MDA (nmol /ml)
Control (Group I)	2.94 ± 0.34*	9.48 ± 2.6*
Diabetic (Group II)	6.23 ± 1.0	22.84 ± 3.8
Diabetic + 100 mg/kg *V. album* (Group III)	4.2 ± 0.82*	12. 22 ± 2.9*
Diabetic + 200 mg/kg *V. album* (Group IV)	3.36 ± 0.89*	10.59 ± 3.0*

*Significantly different from the diabetic (p<0.05)

Figure 4

Table 4: Mean activities of liver of enzymic antioxidant in both experimental and control rats.

Treatment	Liver SOD (units/mg protein)	Liver CAT (units/mg protein)
Control (Group I)	5.63 ± 0.49*	72.21 ± 5.8*
Diabetic (Group II)	2.98 ± 0.37	43.9 ± 3.7
Diabetic + 100 mg/kg *V. album* (Group III)	4.53 ± 0.63*	60.75 ± 3.0*
Diabetic + 200 mg/kg *V. album* (Group IV)	5.04 ± 0.97*	62.38 ± 4.52*

*Significantly different from the diabetic (p<0.05)

Figure 5

Table 5: Mean values of plasma and liver non- enzymic antioxidant in experimental and control rats.

	GSH, (mg/dl)		Vitamin C (mg/dl)		Vitamin E (mg/dl)	
Treatment	Plasma	Liver	Plasma	Liver	Plasma	Liver
Control (Group I)	21.2 ± 1.4*	40.1 ± 3.3*	1.42 ± 0.4*	1.21 ± 0.09*	1.69 ± 0.3*	0.93± 0.05*
Diabetic(GroupII)	12.09 ± 0.8	20.9 ± 2.5	0.71 ± 0.26	0.62 ± 0.05	0.90 ± 0.12	0.50 ±0.02
Diabetic + 100 mg/kg *V. album* (Group III)	18.0 ± 0.7*	33.8 ±2.8*	1.19 ± 0.35*	1.17 ± 0.07*	1.50±0.21*	0.80 ± 0.03*
Diabetic + 200 mg/kg *V. album* (Group IV)	18.8 ±0.6*	36.7 ±3.4*	1.25 ± 0.31*	1.28 ±0.12*	1.58±0.17*	0.84 ±0.4*

*Significantly different from the diabetic ($p<0.05$)

CORRESPONDENCE TO

Harrison Ugo Nwanjo,Ph.D, Department Of Medical Lab. Science, Imo State University, Owerri, Nigeria. E-Mail: Harrisonnwanjo@Yahoo.Co.Uk GSM-08033525389

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Author Information

H. U. Nwanjo
Department of Medical Laboratory Science, Imo State University