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Analysis of certain biochemical indices on alloxan induced diabetic rats administered with protein isolated and purified from *Vernonia amygdalina*

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Abstract

This study investigated the effect of protein isolate from leaf extract of *Vernonia amygdalina* in diabetic rats. Thirty (30) adults male Wistar rats were randomly divided into six (6) groups of five (5) each based on their body weight. Diabetes was induced with administration of alloxan, 150 mg/kg body weight (i.p). Group A served as the control and received 1 mL/kg body weight of 5% ethanol being solvent used, Group B received 1 mL alloxan containing 150 mg/kg only. Group C, D, E and F were respectively alloxanized but treated with 1%, 3%, 5% and 7% of protein isolate obtained from *V. amygdalina* leaves for 14 days. On the 15th day, the animals were humanely sacrificed and their liver homogenates were prepared. Standard biochemical procedures were adopted for determination of catalase (CAT), superoxide dismutase (SOD) activities, Malondialdehyde (MDA) and glucose levels. Data were subjected to one-way Analysis of Variance (ANOVA) with Tukey-Kramer multiple comparison post-hoc test using Graph Pad, version 6 software. Results showed that CAT activity in alloxan-induced untreated rats (1.17 µmole H_2O_2) was significantly ($p < 0.05$) lower than observed in control rats (2.10 mole H_2O_2). Treatment with the respective doses recorded comparable values to those observed in control rats. Similar observation was seen with SOD data. Rats treated with 7% protein isolate recorded the most significant ($p < 0.05$) decrease in serum glucose level. The study suggests that the protein isolate possesses anti-diabetic and hypoglycaemic effects on alloxan-induced diabetic rats.

Keywords: *Protein isolates, diabetes, biochemical, hypoglycaemic, alloxanized, liver homogenate*

1. Introduction

The growth of herbal research in the management of diabetes has increased in the past twenty years. Diabetes mellitus is characterized by alterations in the metabolism of carbohydrate, fat and protein and it is caused by a relative or absolute deficiency of insulin secretion and different levels of insulin resistance. It results from both genetic predisposition and environmental factors (Govindappa, 2015). Some chemicals like alloxan and streptozotocin have been used to induce the metabolic disorders in animal models (Modilal and Daisy, 2011).

Alloxan, chemically called 2, 4, 5, 6-tetraoxopyrimidine is the most prominent chemical compound used in diabetogenic research for the induction of Type I diabetes (Etuk, 2010). It has been widely used to induce

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experimental diabetes in animals such as rabbits, rats, mice and dogs with different grades of disease severity by varying the dose of alloxan used (Iranloye *et al*., 2011). Alloxan is a urea derivative that causes selective necrosis to the β-cells of pancreatic islets (Etuk, 2010). Mechanistically, alloxan evokes a sudden rise in the insulin secretion in the presence or absence of glucose and this insulin release occurs for short duration followed by the complete suppression of the islet response to glucose even when high concentrations of glucose is present (Lachin and Reza, 2012). Alloxan also reacts with two thiol sulphydryl groups (-SH) in the sugar binding site of glucokinase and results in the inactivation of the enzyme, thereby forming dialuric acid which in turn generates reactive oxygen species and superoxide radicals (Das *et al*., 2012). Antioxidants like superoxide dismutase, catalse and the non-enzymatic scavengers of hydroxyl radicals have been found to protect against alloxan toxicity (Ebelt *et al*., 2000).

Ethnobotanical information indicated that more than 800 plants including *Vernonia amygdalina* are used as traditional remedies for the treatment of diabetes due to their effectiveness, less side effects and low cost (Rathjod *et al*., 2008). *Vernonia amygdalina* (family, *Compositae*) is a vegetable shrub that is widely spread in East and West African countries (Dharani *et al*., 2010). In Nigeria, it is commonly known as "bitter leaf" because the leaves and stem have a bitter taste when chewed. Its leaves are used as a popular vegetable for soups particularly among the ethnic groups in Nigeria (Okolie *et al*., 2008). Though a number of studies have reported its anti-diabetic role (Akah and Okafor, 1992; Nwanjo, 2005; Abraham, 2007; Ebong *et al*., 2008; Eteng *et al*., 2008; and Erasto *et al*., 2009), as well as antioxidant potentials (Halliwell *et at*., 2005; Frage, 2007; Oboh *et al*., 2008; Xie *et al*., 2008; and Pownall *et al*., 2010), it became crystal clear to X-ray the particular fraction of the plant that may potentiate such pharmacological relevance. Therefore, this study centred on the evaluation of hepatic catalase (CAT) and superoxide dismutase (SOD) activities, formation of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) as well as changes in glucose level in alloxanized rats treated with *Vernonia amygdalina* leaves protein isolate which will contribute to the growing knowledge on the medicinal use of *Vernonia amygdalina* leaf on oxidative stress-induced diabetes.

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus

Plastic cages (Dana Plast Limited, Nigeria), hand gloves, (Maxwell Glove Manufacturing, Malaysia), micro pipette (HumaPette Smart Line, Germany), weighing balance (Shimadzu, TX323L, England), pH meter (MEMAX, India), cannula, centrifuge (Centrifuge 80-3, Labscience, England), water bath (Gallenhamp, BKS-300-010F, UK), refrigerator (LG), universal sample bottles and spectrophotometer (721 Visible Spectrophotometer, PEC Medical, USA) and other analytical graded instrument / devices were used.

2.1.2. Reagents

All reagents used were of analytical grade and included sodium carbonate, sodium hydrogen carbonate, epinephrine and hydrochloric acid purchased from Loba Chemie PVT, Mumbai, India.

2.1.3. Collection of Vernonia amygdalina leaves

Fresh leaves of *Vernonia amygdalina* were harvested from a garden at Winner's Chapel road, off Ihumudumu road, Ekpoma. It was properly authenticated by a Taxonomist at Botany Department, Ambrose Alli University, Ekpoma.

2.1.4. Experimental animals

Thirty (30) male Albino rats were purchased from the Animal house, College of Medicine, Ambrose Alli University, Ekpoma and acclimatized for one (1) week in a well-ventilated plastic cage at the animal house in the Department of Biochemistry, Ambrose Alli University, Ekpoma. They were subjected to diurnal variation in day light and darkness characteristics of tropical rainforest in May. After acclimatization, the administration lasted for fourteen (14) days.

2.2. Methods

2.2.1. Preparation of Vernonia amygdalina leaves protein isolate

The method of Aletor (2012) was adopted for the preparation of leaf protein concentrate with slight modification. The *Vernonia amygdalina* leaves (3500 g) were washed and weighed prior to pulping using mortar and pestle, followed by pressing in cheese cloth to separate the leaf juice. The separated leaf juice was heated in batches at

80–90 °C for about 10 min to coagulate the leaf protein (Adeyeye and Omolayo, 2011). The protein coagulum was separated by filtering through cheese cloth. The filtrate was then sun-dried for 24 h to obtain the leaf protein concentrate. The yield was stored in a sample bottle, labelled and kept in a refrigerator.

2.2.2. Induction of diabetes

Stock solution of alloxan was prepared by dissolving alloxan monohydrate (0.749 g) in 5 mL of 0.9 % normal saline. Diabetes was induced by single intraperitoneal (i.p) administration of 150 mg / kg body weight of the alloxan monohydrate stock (Akinola *et al*., 2012). The rats with blood glucose level greater than 200 mg / dL, two days post-induction, were considered diabetic and used for this research work.

2.2.3. Management of experimental animals

Experimental rats were randomly assigned to six groups of five (5) rats per group. Ethanol (5%) served as the vehicle for all groups and all the treatments lasted for 14 days. Oral administration of 1 mL / kg body weight of different percentage solution of *Vernonia amygdalina* leaf protein were administered to diabetic rats and used for this research work as below for treated group.

Group A: administered 5% of ethanol and served as control
Group B: administered alloxan, 150 mg / kg body weight
Group C: administered alloxan, 150 mg / kg body weight and 1% of VALPI
Group D: administered alloxan, 150 mg / kg body weight and 3% of VALPI
Group E: administered alloxan, 150 mg / kg body weight and 5% of VALPI
Group F: administered alloxan, 150 mg / kg body weight and 7% of VALPI

2.2.4. Sacrifice of experimental animals

The rats were weighed before being sacrificed. The rats were anaesthetized with chloroform after twenty-four (24) hours of last dose treatment and dissected. The liver were excised and homogenised with equal volumes of normal saline. The homogenates were centrifuged at 10,000 g for 10 min and used for the biochemical analysis.

2.3. Biochemical Analysis

2.3.1. Determination of hydrogen peroxide (H_2O_2)

H_2O_2 levels in liver homogenate of the respective samples were assessed according to the method of Wolff (1994). Liver homogenates (50 µL) were added to 250 µL of a mixture of xylenol orange containing 250 µL of Ammonium Ferrous Sulphate (AFS), 100 mmol / L of sorbitol and 25 mmol / L of tetraoxosulphate (VI) acid. The mixture was vortexed and incubated for 30 min at room temperature. Absorbance of the blue-purple complex formed was read at 560 nm against blank in a spectrophotometer (Visible spectrophotometer, PEC medical). Values were extrapolated from a standard calibration curve (Y= 0.0048x, R^2= 0.975) and expressed in µmol.

2.3.2. Determination of Superoxide Dismutase (SOD) activity

The level of SOD activity was determined by the method of Misra and Fridovich (1972). It is based on activity of superoxide dismutase to inhibit the autoxidation of epinephrine generated by xanthine oxidase. For the determination, liver homogenate, 50 µl from the respective samples were added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2), in a test tube 0.3 mL of epinephrine respectively were added, immediately mixed by inversion and changes in absorbance(s) were monitored every 30 sec for 20 min at 420 nm in a spectrophotometer (Visible spectrophotometer, PEC medical), similar steps were done for the blank containing 50 µL of distilled water. Percentage inhibition was calculated from the relationship;

$$\%\ Inhibition = 100 - \left[100 \times \frac{Increased\ absorbance\ per\ \text{min}\ ute\ for\ sample}{Increased\ absorbance\ per\ \text{min}\ ute\ for\ blank} \right]$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50 % inhibition of the autoxidation of epinephrine.

2.3.3. Determination of Catalase (CAT) activity

CAT activity was determined according to the method of Aebi (1984). Liver homogenate, 50 µL was added to 2.95 mL of 19 mM H_2O_2 solution. Then, the mixtures were rapidly inverted to mix. They were placed in a cuvette and inserted into a spectrophotometer (Visible spectrophotometer, PEC medical) and changes in absorbance read every min for 5 min at 240 nm against blank. CAT activity was calculated from the relationship;

$$CAT\ activity = \frac{\Delta A\,240 / \min \times reaction\ volume \times dilution\ factor}{0.0436 \times sample\ volume \times mg\ protein\ per\ ml}$$

2.3.4. Determination of Malondialdehyde (MDA)

The concentration of MDA in liver homogenate was determined using the method of Varshney and Kale (1990). It is based on the reaction of thiobarbituric acid with MDA to form a pink colored complex. Briefly, 0.4 mL each from liver homogenate was mixed with 1.6 mL Tris-KCl buffer containing 0.5 mL of 30% TCA. Subsequently, 0.5 mL of 0.75 % thiobarbituric acid was added to each before incubation at 80°C for 45 min in a water bath spectrophotometer (Visible spectrophotometer, PEC medical). Next, the mixture in the respective test tubes were cooled in an ice bath and centrifuged at 3000 rpm for 10 min. Absorbance of the respective clear supernatants was read against blank at 532 nm from a spectrophotometer (Visible spectrophotometer, PEC medical). MDA formed / mg protein was computed with a molar extinction coefficient of 0.156$\mu M^{-1}cm^{-1}$ (Adam-Vizi and Seregi, 1982).

2.3.5. Glucose estimation

Blood glucose was measured using the Accu-Check® blood glucometer. On each test strip, there is a test area containing reagents. When blood is applied to the test area, the glucose dehydrogenase enzyme reacts with the blood glucose. The substantial chemical reaction changes the color of the test area. The meter registers this color and converts it into a blood glucose value at approximately, 8 sec. The blood sugar values were expressed in mg / dL.

2.4. Statistical Analysis

The data obtained were subjected to one-way Analysis of Variance (ANOVA) and Tukey-Kramer multiple comparison post-hoc test using GraphPad Prism Statistical Software (Version 6). Results were recorded as Mean ± Standard Error of Mean (SEM) of five determinations. Differences were considered statistically significant at 95 % confidence level ($p < 0.05$).

3. Results

The results for respective analyses carried out in this study are presented in Table 1: Hepatic antioxidant activity, lipid peroxidation and changes in glucose levels in alloxanized rats. As shown, CAT activity in the alloxan-induced diabetic group (1.17 µmol H_2O_2 / min) was significantly ($p < 0.05$) lower than observed in the control group (2.10 µmol H_2O_2 / min). Groups treated with different percentage doses of the plant extract (1%, 3%, 5% or 7%) respectively recorded significantly ($p < 0.05$) higher CAT activity compared to as observed in diabetic untreated group. Comparison of the following;

Control vs alloxan + 1% VALPI (*Vernonia amygdalina* leaves protein isolate) extract

Control vs alloxan + 3% VALPI extract

Control vs alloxan + 5% VALPI extract

Alloxan + 3% extract vs alloxan + 5% extract

Alloxan + 3% extract vs alloxan + 7% extract and

Alloxan +5% extract vs alloxan + 7% extract; were not significantly ($p > 0.05$) different.

The results for hepatic SOD activity in rats that were induced with alloxan alone recorded a significantly ($p < 0.05$) lower activity (0.35 unit) when compared with control group (0.84 unit). The respectively treated rats recorded comparable values to those of the control group. Alloxan induction of diabetes recorded very high hepatic MDA generation (38.31 ± 2.04). The least MDA formation on administration of the respective doses was observed with administration of 1% VALPI (16.50 ± 2.17).

The results for hepatic hydrogen peroxide (H_2O_2) level are as shown in column 5 in Table 1. Alloxan induced diabetic untreated rats recorded a significantly ($p < 0.05$) higher H_2O_2 level (82.49 µmol) compared to the normal control group (52.90 µmol). The H_2O_2 levels in the hepatocytes of rats induced with alloxan and treated respectively with 1% (58.00 µmol), 3% (53.71 µmol), 5% (53.60 µmol) and 7% (45.47 µmol) were all significantly ($p < 0.05$) lower when compared to values observed in alloxan-induced untreated rats (82.49 µmol). These values were however significantly ($p < 0.05$) comparable to values observed in the normal control group.

Table 1: Hepatic antioxidant activity, lipid peroxidase and glucose levels in alloxanized rats

	CAT (µmole H_2O_2/min)	SOD (unit)	MDA	Glucose		H_2O_2 (µmole)
				Initial	Final	
Control	2.10± 0.02	0.84±.1.10	8.60±0.05	101.30± 9.14	106.7± 10.04	52.90 ± 0.63
Alloxan alone, ALZ	1.17± 0.01[a]	0.35± 0.03[a]	38.21± 2.04[a]	110.00± 8.33	599.30± 35.35	82.49 ± 0.59[a]
ALZ + 1% VALPI	2.04± 0.01[b]	0.65± 0.02[b]	16.50± 2.17[b]	120.30± 7.13	311.70± 102.90	58.00 ± 0.43[a]
ALZ + 3% VALPI	2.33± 0.08[b]	0.74 ± 0.03[b]	18.52±1.85[ab]	111.70± 4.49	307.00± 19.00	53.71 ± 1.57[a]
ALZ + 5% VALPI	2.33 ± 0.07[b]	0.77 ± 0.05[b]	28.33± 4.10[a]	108.30± 3.93	114.50± 39.50	53.60 ± 0.14[a]
ALZ + 7% VALPI	2.48 ± 0.10[ab]	0.90± 0.04[b]	24.22± 0.63[ab]	119.30± 3.38	99.33± 8.41	45.47 ± 0.90[a]

Note: Data are presented as mean ± standard error of mean, n = 5; a = Values differ significantly from control ($p < 0.05$); b = Values differ significantly from alloxan only ($p < 0.05$); ab = Values differ from control and alloxan alone; VALPI = Vernonia amygdalina leaves protein isolate; and SOD unit = one unit is given as the amount of SOD that caused 50% inhibition of autoxidation of epinephrine.

4. Discussion

Diabetes is a chronic disorder affecting carbohydrate, fat and protein metabolism (Gajera *et al*., 2008) It is characterized by hyperglycaemia which reflects impaired carbohydrate utilization resulting from defective insulin secretory response (Volk and Arguilia, 2005). This is reflected in glucose level (599.30 ± 35.35 mg / dL) observed in rats induced diabetes with alloxan, 150 mg / kg body weight. This results from two distinct pathological effects of alloxan. Firstly, it selectively inhibits glucose-induced insulin secretion through specific inhibition of glucokinase which is the glucose sensor of the beta cell. Secondly, it causes a state of insulin-dependent diabetes through its ability to induce ROS formation which results in the selective necrosis of beta cells (Szkudelski *et al*., 1998).

Administration of the respective percentage of the isolate resulted in significant ($p< 0.05$) decrease in blood glucose level after a period of 14 days. Mazumder *et al*. (2009) observed similar effects in diabetic rats administered *V. amygdalina* leaf extract. The results confirm alloxan as a useful substance to induce diabetes (Szkudelski *et al*., 1998). Insulin is a hormone produced by the beta cells of the islet of Langerhans of the pancreas. It is responsible for the transfer of blood glucose into cells to be metabolised. It initiates the action by binding to a glycoprotein receptor on the surface of the cell. The receptors consist of an alpha subunit which binds the hormone and a beta subunit, an insulin stimulated tyrosine specific protein kinase. It is the activation of this kinase that is believed to generate a signal that eventually results in insulin action on glucose, lipids and protein metabolism reflected in low blood glucose on administration of the respective doses of protein isolate. This evidence hence, implicates the protein isolate to have the capacity to stimulate tyrosine specific protein kinase in the diabetic rat. This will account for the uptake of glucose in blood in the treated rats to a lower serum level compared to untreated group. It was observed that administration of 7% VALPI resulted in lowest level of glucose in serum (99.33 mg / dL) compared to that observed in control rats (106.7 mg / dL). Hypoglycaemia is characterized by a reduction in plasma glucose concentration and can induce symptoms or signs such as altered mental status or sympathetic nervous system stimulation (Cheesebrough, 1999).

It has been observed that insulin dependent diabetes is associated with induction of Nitrogen Oxygen Species (NOS) and Reactive Oxygen Species (ROS) formation (Heikkila *et al*., 1976). This is reflected in the high level of hydrogen peroxide, 82.49 ± 0.59 µmol observed in alloxan induced diabetic rats compared to 52.90 µmol observed in control. In this study as shown, treatment with / or administration of the *Vernonia amygdalina* leaves protein isolate tended towards restoring the level back to the threshold in the control group, 52.90 µmol. Antidiabetic and antioxidant activities of methanolic extracts of *V. amygdalina* leaves in induced diabetic rats was recently reported by Adeoye *et al*. (2007). This study seems to provide a corroborative evidence in the potential of *V. amygdalina* leaf extract to provide beneficial effect in diabetic state.

Oxidative stress has recently been recognized as a significant player in the pathogenesis of gastro-intestinal complications of diabetes (Kashyap and Farrugia, 2011). It damages cells and results in secondary complications

in diabetes mellitus. This results from damages to cellular protein and lipid that eventually lead to cell death. Consequently, upon peroxidation and release of malondialdehyde, hydrogen peroxide and hydroxyl radicals damages gastric mucosa (Bompella et al., 1991; and Chandrasekharan et al., 2001). The results of this study implicate the protein isolate to have beneficial effects in ameliorating secondary complication associated with gastrointestinal tract in diabetic state. This is reflected in the decreasing effect on hydrogen peroxide level. The level of MDA in alloxan diabetic untreated rats confirms lipid peroxidation and oxidative stress associated with diabetes mellitus (Ceriello, 2000; and Akinosun and Bolajoko, 2007). The results of administration of the different doses suggest protective effects of protein isolate of *V. amygdalina* in diabetic state. This is collaborated with SOD result.

From SOD result, allloxan-induction of diabetes results in a significant ($p< 0.05$) reduction in the activity of superoxide dismutase enzyme activity in the hepatocytes of rats while treatment with various doses of VALPI (1, 3, 5 and 7%) respectively tended to restore it to the status of the control as shown in Table 1. Adeoye *et al*. (2017) documented similar trend in the intestines of rats where they posited that SOD enzyme activity was decreased in the intestines of alloxan induced rats to threshold value of the control rats via treatment with methanolic leaf extracts of *V. amygdalina*. Superoxide dismutase is a powerful antioxidant enzyme that plays crucial roles in promoting health by forming part of our body's primary system of defence against free radical damage (Agbator and Ossai, 2014). In this study, the observed decrease in SOD activity could have resulted from inactivation by $H_2O_2^-$ or by glycation of the enzyme, which has been reported to occur in diabetes (Sozmen *et al*., 2001) as a result of depletion and owing to excessive use or usage of the enzyme to mop up the hyperglycaemia induced free radical generated. Similar observation is reflected in the catalase activity. Catalase is a hemeprotein found in nearly all organs of living organisms (Chelikani *et al*., 2004). The enzyme decomposes $H_2O_2^-$, a powerful harmful oxidizing agent. The significant ($p< 0.05$) reduction in CAT activities in the diabetic control group (1.17 µmole $H_2O_2^-$consumed / min) may be due to the excessive generation of superoxide anion (O_2^-) in diabetic state leading to inactivation of the enzyme (catalase) as O_2^- has been shown to reduce catalase activity (Kono and Fridovich, 1982; and Das and Vasudevan, 2005). Similar observation was made when induction of diabetes with 150 mg / kg of alloxan in rabbits by Owolabi *et al*. (2011) conversely too, administration of the different doses of the protein isolate, (1, 3, 5, and 7% respectively) significant ($p< 0.05$) increase in CAT activity compared to normal control rats were observed as reported in the study of Owolabi *et al*. (2011).

5. Conclusion

This study suggests that *V. amygdalina* leaf protein isolate holds great promise in the development of new therapy(ies) for ameliorating hyperglycaemia induced oxidative stress as occurred in type I diabetic state or alloxan induced oxidative stress. It has beneficial potency in providing free radical scavenging and antioxidant capacity to combat deleterious effect of the condition.

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