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Original Research Article

Assessment of some biochemical indices in alloxan induced diabetic rats treated with protein isolate from Vernonia amygdalina leaf

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Abstract

Vernonia amygdalina leaf is commonly used in traditional medical practice for the management of several disease conditions. This study investigated the effect of administration of the leaf protein isolate (VALPI) on some serum and liver biochemical indices - Glutathione peroxidise (GPX) and reductase, Alanineaminotrasnferase activity, serum total protein, cholesterol, and albumin. Thirty male albino rats were used in the experiment and placed into 6 groups of 5 rats each. Group A served as control. Group B contained rats given intra-peritoneal dose of alloxan that induced diabetes. Group C, D, E and F were as group B but administered line of different percentage solution - 1%, 3%, 5% or 7% respectively of VALPI for 14 days. On the 15th day the rats were euthanized, liver exercised and weighed portion homogenised in equal volume of saline. Standard protocols were used to assay for the biochemical analytes in serum and liver homogenate. Some functional properties of VALPI were assessed with standard methods. Data were subjected to single analysis of valence (ANOVA) with Tukey Kramer multiple comparison post-hol test using Graph pad, version 6 software. Mean significance were considered at 95% confidence level. Haemagluttin activity was not detected in the VALPI which also recorded low in-vitro digestibility, 34.00%+1.16. Comparable GPX activity were observed in test, control, alloxanized and not treated rats,ca257.00 + 0.01 µgGPx/ml. Comparison of other analytes in alloxanized and treated rats were significally (P<0.05) different and suggested beneficial role for administration of VALPI to diabetic rats.

Keywords: **Leaf protein isolation, Euthanized, Biochemical, Analyte, Alloxanized.**

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by persistent hyperglycaemia associated with defects in insulin secretion and insulin in-action or both (Campbell, 2013). It is predicted that the number of persons with the condition is going to rise from 366 million in 2011 to 552 million by 2030 (Whiting et al., 2011). Several pathogenic processes have been linked with diabetes such hypoinsulinemia, deregulated carbohydrate and lipids metabolism, formation of advanced glycation end products (Mahgoub and Abd-elfattah, 1998) and oxidative stress (Baynes, 1991)

Increasing evidences suggest that oxidative stress plays major role in the pathogenesis of diabetes mellitus (Cerriello 2000). This result from persistent hyperglycaemia in diabetes induced by reactive oxygen species (ROS) production by glucose auto-oxidation (Bonnetont- Rousselot, 2002), activation of protein kinase c (Lee et al., 2004) and increased flux through the hexose-amine pathway (Brown Lee, 2005). This excess generation of free radical damages the ß-cells through

the induction of apoptosis (Cnop et al., 2005). As a result, the antioxidant defense present in the system like reduced glutathione (GSH) level may not be adequate to neutralize the excess ROS produced or its synthesis may even be inhibited.

The treatment of diabetes mellitus so far involves the use of anti-diabetic drugs and administration of more insulin to enable the diabetic attain nearly normal carbohydrate, fat and protein metabolism (Nelson and Cox, 2005). Recently, alternative therapy including the use of several medicinal plants has been recommended, especially in places where accesses to conventional management procedures are inadequate. The usefulness of plants to man is not only as a source of raw materials for industries but also as a source of food and medication (Erasto et al., 2006). Several herbal preparations from different parts of plants have become popular for the management of a variety of diseases like diabetes that afflict man and other animals (Pinto and Rivlin, 1999). Plants have been known to possess abundant phytochemicals, antioxidants, antimicrobial and pharmacologically active principles like anthraquinones, flavonoid, saponins, polyphenols, tannin and alkaloids (Sofowora, 2006; Evans, 2005). One of such plants suspected to possess medicinal value is the bitter leaf-Vernonia amygdalina.

Vernonia amygdalina is a perennial shrub commonly known as bitter leaf and belong to the family of Asteraceae (Iwolokun et al., 2006). A number of experimental findings have presented *Vernonia amygdaliris* leaf extract as possessing anti-pathogenic and other beneficial actions (Kupchan et al., 1969; Jisaka et al., 1993; Ijeh et al., 1996; Akinpelu 1999); antioxidant (Torel et al., 1986; Igile et al., 2008) hepato and nephro-protective effect (Ijeh and Obidoa, 2004; Iwalokun et al., 2006). Reports are also available implicating bioactive peptide of aqueous leaf extract of *Vernonia amygdalina* in potentiating anti cancer acton (Izevbigie, 2003; Izevbigie et al., 2004).

This study investigated transaminase activity, reduced glutathione state and gluthation per oxidise, total protein and albumin level in alloxan induced diabetic rats administered Veronia amagdalina leaf protein isolate (VALPI). The effect on total serum cholesterol level was also investigated. Also assessed is the invitro didestibility and some functionality properties of the VALPI used. The essence is to provide an overview in these regards of the possible contribution(s) the leaf protein isolate can make in management of type I diabetic state in an animal modelled perspective.

MATERIALS AND METHODS

a) Reagents: All reagents used were of analytical grade (Loba chemie PVT, Mumbai India; Guangdong Guamghua Sci-Tech Co Ltd China).

b) Apparatus: Plastic cages (Dana plastic limited, Nigeria) Hand gloves (Maxwell Glove Manufacturing, Malaysia) Micro pipette (Humapette smart line, Germany) Weighing balance (Shimadzu TX323L England) centrifuge (Centrifuge 80-3, Lab science, England), Spectrophotometer, 721 visible spectrophotometer PEC medical USA and other analytical grade instruments and devices.

c) Collection of *Vernonia amygdalina* leaves: Fresh leaves of *Vernonia amygdalina* were harvested from a garden at Winner's Chapel road, off Ihumudumu road, Ekpoma, Nigeria. Authentication was done by a Taxonomist, Botany department, Ambrose Alli University, Ekpoma Nigeria.

d2) Preparation of leave protein isolate: The method of Aletor (2012) was adopted for the preparation of leaf protein concentration/ isolate (VALPI) with slight modification and reported earlier (Ihimire et al 2019).

d3) Processing of Red blood cell: Blood was obtained from rabbit after dissection (Rowett., 1977) by cardiac puncture into sterile 1% sodium citrate physiological saline. The tube containing the mixture was centrifuged at 300rpm for 40 minutes. Cells obtained were made up to twice their volume with 1% sodium citrate saline and recentrifuged. This was repeated further thrice to obtain 50% suspension from which 3% required for heaemaglutination test was prepared.

e) Induction of diabetes: Diabetes was induced by single intra peritoneal (IP) ingestion of 150mg/kg body weight of all oxen monohydrate, 01.749g in 5ml of 0.9 saline to rats (Akinola et al 2012) Those with blood glucose level greater than 200 mg/dl two days post induction were considered diabetic and used for the study.

f) Management of experimental animals: Experimental rat were randomly assigned to six groups of five (5) rats per group: Ethanol (5% aqueous) served as the vehicle for all groups. Treatment lasted for 14 days. Oral administration of 1ml/kg body weight of different percentage solution of Vernonia amygdalina leaf protein isolate (VALPI) was used for treatment.

GROUP A: Administered 1ml of 5% ethanol and served as control.

GROUP B: Administered alloxan, 150mg/kg body weight (IP).

GROUP C: Administered alloxan, 150mg/kg body weight (IP) and 1ml of VALPI each day.

GROUP D: Administered alloxan, 150mg/kg body weight (IP) and 1mlof VALPI each day.

GROUP E: Administered alloxan, 150mg/kg body weight (IP) and 1mlof VALPI each day.
GROUP F: Administered alloxan, 150mg/kg body weight (IP) and 1mlof VALPI each day.

g) Sacrifice of experimental animals: Respective rats were weighed, anaestisized with chloroform after twenty four (24) hours of last dose treatment and dissected (Rowett., 1977). Blood was collected by cardiac puncture. Standard method was used to process the blood to obtain serum .Their liver were excised and homogenized with equal volume of normal saline. The homogenates were centrifuged as 10,000rpm for 10 minutes and the supernatant was used for all assays.

Determination

Functional properties: The method of Xiaoyirig and Hua (2012) was adopted for determination of water absorption capacity, oil absorption capacity, foam capacity and emulsifying capacity. All results were expressed in percentage.

In vitro Digestibility: Modified procedure of Lamanu et al. (2011) was used. Sample (0.5g) was suspended in 50ml 0.1M Phosphate buffer pH 7. Subsequently 5ml of 11% trypsin solution was added and left for 30 minutes. The crude protein CP immediately the enzyme was (CP1) added and after 30minutes on addition, CP2 were determined with buiret reagent. Percentage in vitro digestibility was calculated from the relationship.

$$\frac{cp1-cp2}{cp1} \times \frac{100}{1}$$

Processing of Red Blood Cell (RBC): The procedure reported by Campbell and Sanford, 1939 was used. Blood obtained by cardiac puncture from rabbit was added to thrice its volume of sterile 1% sodium citrate physiological saline solution. Solution was centrifuge at 3000 rpm for 40minutes. The RBC was collected and further treated as above twice before dissolving the corpuscle into equal volume to the same physiological saline to obtain a 50% suspension from where the 3% required for haemaglutination test was prepared.

Haemaglutination Test: The procedure reported earlier in literature was followed (Crichton and Walker, 1985; Kakade *et al.,* 1974). It was done with 40% w/v mixture of finely grounded sample protein isolate (425µml) in 0.1M phosphate buffered saline (PBS) PH 7.4. A drop of this was added to droplets of RBC in a 96 well plate. The mixture was rocked at room temperature on a mechanical shaker for 10minutes. Reaction were recorded on weak (+), moderate(++), strong (+++) positive or Negatives (-)

Biochemical assays: The method described by Jollow et al. (1974) was adopted in the evaluation of reduced Glutathione. Alanine aminotransferase (ALT) level was determined following the principle described by Reitman and Frankel (1957). It involved assessing the arte of formation of pyruvate by coupling the Alanine aminotransferase reaction with that lactate dehydrogenase which converts the pyruvate to lactate the decrease in absorbance ayt 340µm was measured a NADH is oxidized to NAD

PYRUVATE + NADH —> LACTATE + NAD

Gamma glutamyl transferase (GGT) activity was assayed with a modified Szasz procedure (Szasz, 1969). The substrate employed in this method is Gamma-glutamyl-p-niltroanilide(GGPNA) glyceylglycine served as an acceptor and tris (hydrogxy methyl) aminomethaneprovided buffering effect, serum was added to the buffer acceptor solution and the rection initiated by adding substrate in dilute hydrochloric acid solution. An appropriate blank was used to correct for hydrolysis. The increase in absorbance at 405µm due to the p-nitro aniline formed was measured spectrophotometrically at 30℃. Total protein and Albumin were determined according to Buiret method. (Grant and Kach mar., 1982) In the buiret reaction one copper ion is linked to between 4 α 6 nearby peptide linkage by coordinate bond. The intensity of the colour produce is proportional to the number of peptide undergoing reaction. The measurement of albumin was based on its quantitative binding to the indicator 3,3,5,5[1] tetrabromo-m-cresol sulphone-phthalein i.e. bromocresol green BCG (Grant et al., 1987) Glutathione peroxidase activity (GPx) was determined with the method of Rotruck et al (1973). The method reported by Kaplan and Szabo (1979) was used to assess total cholesterol.

I) Statistical Analysis: Data obtained were subjected to one-way analysis of variance and Turkey Kramer post hoc test using Graph Prism (Version 6) software. Results were recorded as mean ± standard error of mean of triplicate assay results. Significant difference was considered al 95% confidence level.

RESULT

The results from this study are presented in Table 1 and 2.

Table 1 as shown below contain some physico-chemical characteristics of the VALPI. As shown haemaglutination activity was not detected in the VALPI used for study.

The *in vitro* digestibility was low as well as foam capacity. Water or oil absorption was above average in

Table 1. *In-vitro*-Digestibility and Functional Properties of VALPI.

In-vitro digestibility %	34.00±1.16
Haemagluttin activity	Nil
Water absorption capacity	89.83±3.86
Oil absorption capacity %	60.43±15.47
Foam capacity %	26.67±5.16
Emulsifying capacity %	57.00±2.37

Result: Mean±standard error of mean for triplicate analysis.

Table 2. Some serum analyte level and some liver homogenate enzyme activities.

Group	GGT IU/L	ALT IU/L	Glutathione µgGSH/ml	Glutathione µgGPx/ml	Total Protein TPg/dL	ALB g/dL	Total Cholesterol ml/dL
Control (A)	l.54±0.39	98.01±1.30	35.39±039	257.10±0.03	7.16±0.25	3.62±0.38	103.80±1.51
Alloxan alone (B)	2.32±0.67	106.30±0.95a	29.43±0.26a	257.10±0.01	5.43±0.36a	3.53±0.12	109.30±4.09
ALZ + 1% VALPI (C)	2.32±0.67	103.20±0.50a	32.77±0.12b	257.10±0.03	6.11±035b	4.11±0.22	100.20±0.21
ALZ + 3% VALPI (D)	1.16±0.00	114.10±1.13ab	33.48±0.19b	257.10±0.07	6.62±0.16b	43.79±0.30	98.32±0.42b
ALZ + 5% VALPI (E)	1.16±0.00	99.70±0.56b	43.26±0.94ab	257.10±0.05	6.85±0.03b	4.62±0.48	94.98±1.11b
ALZ + 7% VALPI (F)	1.16±0.00	99.07±0.29b	37.52±1.10b	257.10±0.02	6.53±0.11b	4.29±0.09	96.87±1.47b

Result: Mean±standard error of mean for triplicate analysis. Values in the same columns with different alphabetic superscript are considered statistically significant (P<0.05).

percentage while emulsifying capacity was slightly above average in percentage also.

Table 2 as shown above contain some biochemical indices assessed in the test rats or control.

Statistical significant (P<0.05) difference in analyte values were observed. For total protein Group B recorded significantly (P<0.05) greater values than observed in Group A. For cholesterol level groups administered 3% VALPI or 5% VALPI or 7% VALPI respectively recorded significantly (P<0.05) lower values compared to that observed in Group B. The level of ALT in Alloxan + 1% VALPI or Alloxan 1150mg/Kg only groups respectively were significantly (P<0.05) greater than observed in control. The value observed in Alloxan + 3% VALPI when compared with value observed in Alloxan (150mg/Kg) only group were comparable. Reduced glutathione observed in Alloxan (150mg/Kg) only group was significantly (P<0.05) lower than observed in control group. All test group recorded significantly (P<0.05) higher reduced glutathione level compare to as observed in Alloxan (150mg/Kg) only group. Comparable GGT values were observed in test-and control group of studied subjects.

Induced - diabetic rats that were not treated recorded significantly (P<0.05) lower total protein 5.43 + 0.036 g/dl compare to as observed in control rats, 7.61+0.25g/dl. Total protein level respectively in induced-diabetic rats treated with VALPI were significantly (P<0.05) higher than observed in the untreated diabetic rats. The albumin level in diabetic rats were comparable in the respective rats treated with VALPI.

DISCUSSION

Functional properties of foods concern any non-nutritional properties that affect its utilization (Onimawo and Akubor 2012). The VALPI used for this study recorded high water absorption capacity as two types of ready to eat supplementary formulation. Measurement of water and oil absorption provide data for selecting raw food protenious materials as they provide valuable information on hydration, swelling, solubility and geletion (Onimawo and Akubor 2012). The high water absorption capacity of VALPI suggests that it is capable to imbibe a disproportional amount of water in a food/feed mixture and cause dehydration of other components in the system.

Low percentage in-vitro digestibility, 34.00±1.16 was observed with VALPI used in the study compare to 58-70 reported for germinated oat at different stages (Qin and Jian-Guo 2015). Difference in digestibility could reflect influences of proteolytic enzymes, digestion conditions as well as the status of protein source (Abdel-Aal 2008). Negative agglutination activity was observed as seen with *ocimum basilicum* investigated under similar condition using human blood group (Ahmad et al 2016). This suggests that usage of the product for food/feed will not have adverse effect on blood. But haemaglutinins or phyto-lectins when present in sample do provide a cheap source of blood typing reagent (Wong et al., 2010).

The study revealed that administration of alloxan, 150mg/kg body weight significantly ($P<0.05$) lowered reduced glutathione activity in untreated subject. This observation can be accounted for by the dual consequences of alloxan administration or induction of diabetes. It leads to formation of dialuric acid that is re-oxidized to establish redox cycle. This generates reactive oxygen species –ROS and superoxide radicals (Das et al 2012). Reduced glutathione has been reported to play key role in redox signalling (Lu 2013) believed to be vital in detoxification of xenobiotics, modulation of cell proliferation, apoptosis, immune functioning and fibro-genesis. Hence the low level observed in the alloxanized untreated group is a reflection of the diabetic state resulting from administered alloxan. This is because persistent hyperglycaemia as established with alloxan induction generates reactive oxygen species (ROS) via glucose autoxidation (Bonnfront-Roussel, 2012); activation of protein Kinase C (Lee et al., 2004) and increased flux through the hexosamine pathway (Brownlee, 2005). These affected the reduced glutathione level and could have contributed to the low level of reduced glutathione observed in the group that was alloxanized. Ramkumar et al. (2013) studied the protective effect of alloxan induced oxidative stress in rats and reported a lower reduced glutathione level in liver of alloxan induced diabetics rats compare to as occur in control as observed in this study. The level of reduced glutathione observed in alloxanized rats and the respectively administered 1%, 3%, 5% or 7% VALPI implicate the extract as a potent antioxidant agent that can mitigate against assault on reduced glutathione in diabetic insult resulting from generation of ROS.

The level of glutathione peroxidase activity (GPX) observed in control, untreated and test group in this study were comparable. Owolabi et al 2010 reported significant increase in serum glutathione peroxidase activity of alloxan induced diabetic rabbits treated with 400mg/kg body weight of aqueous leaf extract of *vernonia amygdalina.* This was accounted for by prevention of glycation and in activation of enzyme by high reactive oxygen species and hydrogen peroxide production.

Diabetes mellitus has been linked with liver damage (Yuniartu and Lukiswanto, 2016) and is normally associated with high ALT value. This is reflected in the significantly ($P<0.05$) higher ALT value observed in alloxanized untreated rats, 106.30±0.95 compare to that observed in control, 98.01±1.30. This high value collaborate suggested existence of medical problems such diabetes, viral hepatitis, congestive heart failure, bile-duct problem, infection mononucleosis or my apathy, found in elevated serum ALT levels (Paul and Giboney, 2011). Hence the value confirms the capacity of alloxan to induce diabetes.

Treatment with VALPI, specifically (5% or 7%) reduced the ALT level to comparable status as in control rats. Momoh et al. (2014) had earlier reported similar trend. This suggest that the extracts (VALPI) has ameliorating effect and can contribute positively in liver damage associated with diabetes (Yuniartu and Lukiswanto, 2016).The statistically significant differences observed with levels of different doses administered suggest that complete ameliorative ability of VALPI against liver damage can be achieved at high dose than 5% or 7% or on administration of these for a longer period than did in this study.

Gama glutamyl transaminase, GGT, catalyzes the transfer of gamma-glutamyl functional groups from molecule such as glutathione to an acceptor like amino acid, a peptide or water to form glutamate (Tate & Meister 1985, Whitfield 2001). All rats alloxanized but untreated and those administered 1% VALPI recorded comparable GGT activity 12.32±0.67 but significantly ($P<0.05$) higher than observed in control group. Rats in group administered other doses - 3%, 5% or 7% respectively when compared to those in control group suggest that administration of VALPI makes contribution to lower GGT. GGT is involve in the transfer of amino acids across cellular membrane, in leukotriene metabolism and transfer of glutamyl moiety to a variety of acceptor molecules including water, certain L - amino acids and peptide (Meister 1974, Rault et al, 1985). This leaves the cysteine product to preserve intracellular homeostasis of oxidative stress (Schulman et al 1975; Yokoyama 2007).This reflect the capacity of VALPI to play significant role in oxidative stress in intracellular homeostasis. It has earlier been observed that the VALPI contain peptide/amino group. This could have contributed to reduction of GGT activity by providing alternate substrate source. Also the VALPI as shown earlier is a high water absorbent hence can make this easily available for GGT activity that could have also lower the value.

In this study high cholesterol in alloxanized untreated rats were observed compare to values observed in control group. Administration of the respective doses of VALPI recorded low serum cholesterol level. This study suggests that the VALPI used made enhanced contribution that increase synthesis of bile juice which

aided lipids digestion and metabolism to support the diabetic state (Jessie et al., 2017). This can account for the lowered level of level of cholesterol observed in the study.

Alloxan-induction of diabetes as could be seen in Table 1 led to a significantly ($P<0.05$) decrease in serum total protein level. In a previous study conducted by Obia et al. (2017) a similar observation was reported and was associated with hyperglycaemia and abnormal fat metabolism. The lower level is hence a confirmation of diabetic state of the experimental animals and a reflection of the efficacy of alloxan in inducing diabetes.

Albumin content in alloxanized untreated rats was low compared to as observed in control group. Albumin is the most abundant plasma protein (wang et al, 2015). It is involve in many physiological processes including maintenance, colloidal osmotic pressure, binding and transport of substances acting as amino source. It has been reported that cessation of insulin treatment in diabetic rats lead to a significant reduction in the synthesis of albumin relative to total protein (peavey et al, 1978). Low level of albumin is also associated with increase glycation of plasma protein especially HbA1C in diabetes (Obia let al*.,* 2017). Albumiruria is a feature of complicated diabetes (Schmidt et al., 199). As could be seen in the table administration caused increased level of albumin. And hence since maintaining the serum concentration of albumin is a contributory factor in diabetic management, the study implicate VALPI as a potent efficacious agent in the management of diabetes. This role was met and reflected in the level of albumin in rats administered the respective doses of VALPI.

CONCLUSION

This study has articulated contributory beneficial roles of protein isolate from bitter leaf (*Verninia amygdalina)* to health in animal mode. It enhances bile juice production which could aid in lipids digestion and metabolism. It enhances the synthesis of albumin hence reduces the risk in type 2 diabetes and complication of diabetes in general. Evidence from ALT, or and glutathione peroxidase point to ameliorating effect of VALPI in diabetic management.

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