EFFECT OF ETHANOLIC ROOT AND TWIG EXTRACTS OF VERNONIA AMYGDALINA (ETIDOT) ON LIVER FUNCTION PARAMETERS OF STREPTOZOTOCIN INDUCED HYPERGLYCAEMIC AND NORMAL WISTAR RATS

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Abstract

The effects of various concentrations of ethanolic extracts of *Vernonia amygdalina* (VA) stem and root on some biochemical indices of liver function in streptozotocin-induced diabetic and non-diabetic rats treated with 200mg/kg b.w and 400mg/kg b.w was determined. LD50 value of 1000 mg/kg was established. The oral administration of the extracts lasted for 28 days. The rats were divided into five parallel groups of non-diabetic and diabetic rats, with six rats in each group. Group 1 received Dimethy sulfoxide(DMSO) and served as the negative control. Group 2 received 200mg/kg b.w *Vernonia amygdalina* (stem), group 3 received 400mg/kg b.w *Vernonia amygdalina* (stem). Group 4 received 200mg/kg b.w *Vernonia amygdalina* (root). Group 5 was given 400mg/kg b.w *Vernonia amygdalina* (root). Both stem and root extracts (200 and 400) mg/kg b.w significantly improved body weight and reduced whole blood glucose levels to normal but not up to hypoglycaemic level in diabetic treated rats. Total protein and albumin in serum were increased in diabetic rats treated with stem (200 and 400) mg/kg b.w respectively. The findings suggest that *V. amygdalina* ethanolic stem and root extracts is hepatoprotective in diabetic rats and may be of nutritional/ clinical   relevance amongst African populations for treatment of different ailments.

Keywords: *Vernonia amygdalina*,biochemical indices, diabetes and Wistar rats

Introduction

Streptozotocin (STZ) is a drug currently used to induce diabetes in animal models that resemble non obese type 2 diabetes mellitus (Nakamura *et al*., 2006).The chemistry of STZ describes a glucose moiety with highly reactive nitrosourea side chain. The glucose moiety direct its uptake by pancreatic beta cells with ease via binding to a membrane receptor recently identified to be glucose transporter 2 (GLUT2) (Thuselen *et al*., 1997). The nitrosourea side chain on the other hand is thought to initiate its cytotoxic action. The induction of diabetes with STZ increases the production of free radicals that damage the pancreatic DNA and thus affect insulin secretion (Oguni *et al*., 2003). This is achieved by depleting Nicotinamide adenine dinucleotide (NAD) which is a substrate of poly adenosine diphosphate(ADP) ribose synthetase, an enzyme which is used in DNA repair.

In diabetes, lipid abnormalities, anaemia, alteration of liver and kidney functional indices have been implicated as major risk factors to the progression of microvascular and macro vascular complications (Mandade and Screenivas, 2011).

The promotion of traditional African diets still remains a powerful weapon in the fight against diabetes.Different types of antidiabetic drugs such as biguanide, sulphonylurea, along with insulin has been employed for the treatment of diabetes. Still none of these drugs were able to cure the diseases without adverse reaction (Tolman and Chandramouli, 2003; Annes *et al*., 2007).

Presently, there is a growing interest in herbal remedies due to the side effects associated with oral hypoglycaemic agent (Kim *et al*., 2006). Consequently, further scientific investigation is needed to validate the usage of these plant parts in accordance with the world health organization (http:// www.who.org)

Vernonia amygdalina(VA) Del. popularly known as bitter leaf or etidot in Efik language, is a shrub of 2-5 m tall with petiolate green leaves of about 6 mm diameter. The leaves are characteristically bitter but the bitterness can be abated by boiling or by soaking in several changes of clean water (Burkill, 1985). The stem and root divested of the bark are used as chewing sticks in Nigeria. More importantly, the leaves are a very popular soup vegetable and have even been reported to be consumed by goats in some parts of Nigeria (Aregheore *et al*., 1998).

All parts of the plant are pharmacologically useful. The roots and the leaves are used in ethnomedicine to treat fever, hiccups, kidney problems

and stomach discomfort among several other uses (Burkill, 1985; Hamowia and Saffaf, 1994). Both aqueous and alcoholic extracts of the stem, bark, roots and leaves are reported to be extensively used as a purgative, antimalarial and in the treatment of eczema (Kupcham, 1971). The plant has acquired special relevance recently, having been proved in human medicine to possess potent antimalarial and antihelminthic properties (Abosi and Raseroka, 2003) as well as antitumorigenic properties (Izevbigie *et al*., 2004).The plant has also an amazing antiparasitic efficacy in zoopharmacognosy as it is easily recognized and used for self medication by parasitized chimpanzees (Huffman, 2003).

Despite these varied uses of the plant, there has been insufficient information on its toxicological potentials on the animal system of diabetic model.

Although, the plant is used traditionally for the management of DM, there is still a need for scientific data to give credence to its usage in folklore medicine. A thorough search of the literature reveals that there is scanty information on the effect of this plant parts (stem and root) on blood glucose and pathophysiologic symptoms in diabetic model. The present study was therefore designed to investigate the effect of the ethanolic extract of *Vernonia amygdalina* (stem and root) on body weight and liver functional indices of STZ diabetic rat and normal rats.

Materials And Methods
Sample collection and preparation
Vernonia amygdalina (VA) plants having been identified and authenticated by a Botanist (Dr Mike Eko) of the Department of Botany, University of Calabar, fresh roots were excavated and stem of the plant were harvested from Endocrine Farm, University of Calabar staff quarters, Cross River State, Nigeria.

The roots and stems were thoroughly washed to remove debris and the earth remains. From these the barks were divested and thereafter chopped into bits and allowed to dry under shade. They were blended into fine powder using a Q-link electrical blender Model QBL-18L40. Three hundred and twenty grams (320) of the blended stem bark and Three hundred and seventy grams (370g) of the blended root barks were separately soaked in 1200ml of ethyl alcohol (80% BDH) each, then they were vigorously agitated and allowed to stay in refrigerator for 48 hours at 4°C. The mixtures were first filtered with cheese cloth, then with WhatMan No 1 filter paper (24cm). The filtrates were then separately concentrated *in vacuo* using Rotary Evaporator (Model RE52A, China) to 10% of its original volume at $37\ ^{o}$C - $40\ ^{o}$C. These were concentrated to complete dryness in water bath, yielding 35.6g (11.47%) of stem bark and 52g (14.40%) of root bark

extracts. The extracts were stored in a refrigerator from where aliquots were used for the administration.

Animal handling

Sixty albino rats (males and females) of Wistar strains weighing 120-180g were used for this study. The rats were obtained from the animal house of the Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar .They were acclimatized for one week then reweighed and housed in wooden cages with wire-mesh top and kept under controlled environmental conditions of temperature ($28 \pm 20C$), relative humidity ($50 \pm 5\%$) and a 12 hour light/dark cycle. The animal facility was adequately ventilated and the animals maintained regularly on the commercial rat chow. Tap water and food were provided *ad libitum* throughout the experimental period

Induction of experimental diabetes

Diabetes was induced by intra-peritoneal injection of 65mg/kg b.w of streptozotocin (STZ), Batch No. U925 obtained from Sigma St. Louis, M.O. USA. Prior to diabetes induction, the animals were fasted for 12 hours. Confirmation of diabetes was done four days after induction (Fasting Blood Sugar), using One Touch Glucometer (Lifescan Inc 1995 Milpas, California, USA). Blood sample for the FBS determination was obtained from tail puncture of the rats, and animals with FBS $\geq$ 200mg/dl were considered diabetic and included in the study as diabetic animals.

Determination of median lethal dose (Ld$_{50}$)

The median lethal dose (LD) of the extract was estimated using albino mice by intraperitoneal (i.p) route using the method of (Lorke, 1983) .

Experimental Design

Schedule for animal grouping and treatment is as shown in Table 2. The dosages of plant extracts used were according to the methods of Atangwho 2007a. Plant extracts were administered twice daily via orogastric intubation for 28 days (at 6.00 am and 6.00pm). At the end of 28 days, feed was withdrawn from the rats and they were fasted overnight, but with free access to water. They were then anaesthetized under chloroform vapours and sacrificed. Whole blood was collected via cardiac puncture using sterile syringes into plain tubes and allowed for about two hours to clot. The clotted blood was centrifuged at 3000rpm for 10mins for serum collection meant for biochemical assays.

Table 1: Experimental Design

| Group	Extracts /Doses	Diabetic(n)	Non Diabetic(n)
(1)	**Control** (0.2ml DMSO)	6	6
	STEM EXTRACT		
(2)	200mg/kg b.w	6	6
(3)	400kg/kg b.w	6	6
	ROOT EXTRACT		
(4)	200mg/kg b.w	6	6
(5)	400kg/kg b.w	6	6

DMSO = Dimethylsulfoxide   n = number of rats per group
b.w = body weight

Glucose Estimation

Randox-assay kit (CHOD-PAP) method based on Barham and Trinder (1972) was used. The principle involves the enzymatic oxidation of glucose in sample by the enzyme Glucose oxidase which generates hydrogen peroxide and gluconic acid. The concentration of H_2O_2 released is proportional to the initial amount of glucose in the sample and it reacts under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red violet quinoneimine dye whose colour intensity reflects the concentration of glucose in the sample.

Alanine aminotransaminase (ALT)

Estimation of alanine aminotransaminase (ALT) activity is done using randox kit based on Reitman and Frankel (1957).

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutarate} + \text{pyruvate}$$
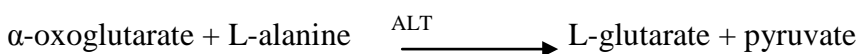
Pyruvate whose concentration depends on the amount of L-alanine transaminated and the activity of ALT is measured by monitoring the concentration of pyruvate hydrazone formed with (2, 4-dinitrophenyl) hydrazine at 546nM.

Aspartate aminotransaminase (AST)

Principle:Estimation of aspartate aminotransaminase (AST) activity is done using randox kit based on Reitman and Frankel (1957).

$$\alpha\text{-oxoglutarate} + \text{L- aspartate} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{oxaloacetate}$$
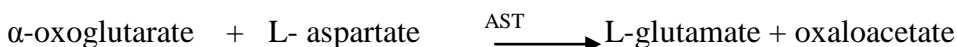
oxaloacetate, with concentration in proportion to aspartate consumed by enzyme and hence its activity is measured by monitoring the concentration of oxaloacetate hydrazone formed with (2, 4- dinitrophenyl) hydrazine.

Alkaline phosphatase (ALP)

Determination of Serum Alkaline Phosphatase activity is done using the method of Baker and Silverton (1985). Absorbances of the samples were read on a spectrophotometer at 400nm against water blank.

Total Protein

Total protein is done using Randox assay kit (Biuret Method) as described by Tietz (1976).

Principle: The method is based on the knowledge that cupric ions in an alkaline medium, interact with protein peptide bonds resulting in the formation of a purple-coloured copper- protein complex which is read colorimetrically at 540nm.

Albumin

Determination of albumin using Randox assay Kit (Bromocresol Green) based on Grant (1987).Albumin measurement is based on its quantitative binding to the indicator, bromocresol green, BCG (3,3',5,5',-tetra bromo- m cresol sulfonpthalein). The albumin-BCG- complex formed absorbed at 578nM and its colour intensity is directly proportional to the concentration of albumin in the sample.

Total Bilirubin

Total bilirubins was determined according to the method of Malloy and Evelyn (1937) as modified by Tietz (1976).Total bilirubin was determined in the presence of caffeine benzoate which releases albumin-bound bilirubin. Bilirubin then reacts with diazotized sulphanilic acid to form pink-coloured azobilirubin which in the presence of alkaline tartrate is converted to green colour.

Statistical Analysis

Glucose and electrolyte measurements are presented as mean $\pm$ SE. One way Analysis of Variance (ANOVA) and the LSD post hoc test were used to analyse the data (p<0.05).

Result
Acute toxicity test

The mean effective dose or medial lethal dose of the extract was investigated using 36 mice on test doses of 100-2000mg/kg body weight. At

the end of twenty four hours in each case, no deaths were recorded within this dose range and no overt manifestation of toxicity was observed up to the dose level of 2000mg/kg body weight. The whole experiment was repeated by gavaging the dose levels again i.e. 3000mg/kg, 4000mg/kg and 5000mg/kg body weight respectively to another set of mice for a period of 24hrs. As was the case in the first test, no deaths were yet recorded nor was overt manifestation of toxicity observed. Therefore, the lower dose level of 200mg/kg and 400mg/kg body weight were chosen as the tolerable doses of the extract.

Table 2(a and b) and 3 (and b) show the effects of administration of *V.amygdalina* stem bark and root bark extract for 28 days on body weight and fasting blood glucose in normal and STZ-induced diabetic rats. Body weight and fasting blood glucose significantly increased in diabetic rats when compared to the non diabetic control. Both stem and root extract (200 and 400) mg/kg b.w significantly improved body weight and brought down whole blood glucose towards normal but not up to hypoglycaemic level.

Weight variation in non diabetic rats of both control and treated groups showed a similar trend weight appreciation at the end of 28th day. However *V.amygdalina* root extract treatment 200mg/kg and 400mg/kg b.w showed smaller increase in weight (reduced increase) compared to other treatment groups and the control. Administration of *V.amygdalina* stem Bark (200mg/kg and 400mg/kg) to non diabetic rats increases the fasting blood glucose and they compared well with their respective non diabetic controls.

Glucose concentration in blood serum of diabetic control animal was significantly increased by about 6.4 fold compared to non diabetic control. Treatment with *V.amygdalina* stem extracts significantly reduces ($p < 0.05$) serum glucose in diabetic treated rats relative to the diabetic control, as non diabetic rats treated with root extracts200mg/kg had serum (8.69±3.24) glucose level reduced significantly ($p < 0.05$) compared to the non diabetic value (36.69±4.28). Serum Amino transferase activities (AST and ALT) were raised by 1.6 and 2.3 fold respectively in the diabetic control relative to non diabetic control.

Treatment with *V.amygdalina* stem 200mgkg and 400mg/kg b.w caused significant reduction $p < 0.05$ in AST and ALT respectively in diabetic treatment groups relative to diabetic control. No significant change was observed in *V.amygdalina* root treated diabetic groups even though there was a reduction in serum values of AST and ALT. Administration of *V.amygdalina* stem (400mg/kg) alone to non diabetic rats caused significant increase ($p < 0.05$) In ALT activity unlike *V.amygdalina* stem 200mg/kg, *V.amygdalina* root 200mg/kg and 400mg/kg b.w caused no significant increase in ALT activity. Alkaline phosphatase (ALP) activity showed no

significant change in both treated diabetic and non diabetic rats administered with *V.amygdalina* root 400mg/kg b.w extract relative to control.

The induction of diabetes in rats significantly (p<0.05) reduced the levels of total protein and albumin when compared to the non diabetic control. The result showed no significant increase in total bilirubin between the various treatment groups. Treatment of diabetic rats with *V.amygdalina* stem extracts at doses of 200 (82.01±0.02) and root extract at doses of 200 (48.01±1.78) increased remarkably (p<0.05) for total protein and albumin respectively. Levels of serum total protein and albumin significantly increased (p<0.05) following treatment with *V.amygdalina* stem 200mg/kg b.w extract in non diabetic rats compared to the non diabetic control.

Table 2a: Effect on treatment on body weight and Fasting Blood Glucose in diabetic rats.

Group Treatment	Body weight		Fasting blood glucose	
	0 day	28th day	0 day	28th Day
Normal control	150.05±0.01	170.02±0.04	56.52±2.86	78.68±3.92
Diabetic control	168.04±0.17	137.01±1.02*	226.25±18.90	246.55±3.34[a]
Stem(200mg/kg)D	169.02±0.09	176.06±0.05*[a]	228.49±2.78	122.51±6.05 [a]
Stem(400mg/kg)D	172.02±0.65	178.06±0.07*	240.00±8.57	95.52±14.42 [a]
Root(200mg/kg)D	165.03±0.09	172.06±0.69	253.67±3.20	119.39±0.94 [a]
Root(400mg/kg)D	162.21±1.45	164.01±2.03*	248.69±2.89	130.058.19 [a]

Values are expressed as Mean ± SD, n=6, *P<0.05 vs NC; a = P<0.05 vs DC
D = Diabetic

Table 2b: Effect on treatment on body weight and Fasting Blood Glucose in non diabetic rats

Group Treatment	Body weight		Fasting blood glucose	
	0 day	28th day	0 day	28th Day
Normal control	150.05±0.01	170.02±0.04	56.52±2.86	78.68±3.92
Stem(200mg/kg)ND	159.25±5.60	182.20±4.50[a]	54.60±4.97	76.20±.80[*]
Stem(400mg/kg)ND	165.90±3.20	186.30±6.90[a]	64.00±0.09	77.00±2.96*
Root(200mg/kg)ND	200.85±10.90	222.48±5.85	69.81±9.61	68.01±0.90
Root(400mg/kg)ND	185.20±6.22	200.60±3.18	58.90±6.80	35.60±5.95

Values are expressed as Mean ± SD, n=6, *P<0.05 vs NC; a = P<0.05 vs DC
NC =normal control, DC =Diabetic control ND=Non diabetic

Table 3a: Effect of treatments on serum glucose and liver function indices in diabetic rats.

Group Treatment	Glucose Mg/dl	ALP (U/L)	AST (U/L)	ALT (U/L)	T.Protein (Mg/dl)	Albumin (Mg/dl)	T.BIlirubin (Mg/dl)
Normal control	36.69±4.28	80.14±6.17	75.07±6.75	26.13±1.71	79.90±0.35	47.9±6.52	7.80±0.90
Diabetic control	233.46±23.40	142.14±0.55	121.84±6.92[a]	61.21±7.47[a]	63.19±0.35[a]	29.44±1.13[a]	43.70±10.80
Stem(200mg/kg)D	107.59±7.25[a*]	98.64±7.51	89.16±8.70[a]	31.43±2.93[a]	82.01±0.02	48.01±1.78	44.30±5.80
Stem(400mg/kg)D	98.04±6.86[a*]	89.74±7.93	85.41±4.60	28.82±1.52[a]	78.52±5.88[a]	59.05±2.50	40.07±0.80

Root(200m g/kg)D	126.21±1 9.26	130.56± 21.90	105.00± 9.70	40.26± 19.8	64.21±1 0.19	38.20± 9.76	38.80±5 .29
Root(400m g/kg)D	116.41±2 1.80	133.68± 15.90	115.40± 2.20	38.72± 16.7	72.80±1 6.5	46.08± 1.08	43.08±0 .90

Values are expressed as Mean ± SD, n=6, *P<0.05 vs NC; a = P<0.05 vs DC

NC =normal control, DC =Diabetic control D = Diabetic

Table 3b: Effect of treatments on serum glucose and liver function indices in non- diabetic rats.

Group Treatment	Glucose Mg/dl	ALP (U/L)	AST (U/L)	ALT (U/L)	T.Protein (Mg/dl)	Albumin (Mg/dl)	T.BIlirubin (Mg/dl)
Normal control	36.69± 4.28	80.14±6. 17	75.07± 6.75	26.13±1. 71	79.90±0. 35	47.90± 6.52	7.80±0. 80
Stem(200mg/ kg)ND	28.70± 9.36	76.48±4. 82	74.89± 1.13	24.33±2. 39	82.40±5. 40*	61.40± 0.04	8.90±9. 70
Stem(400mg/ kg)ND	19.28± 4.45	78.29±1 0.90	71.98± 0.14	25.28±9. 32*	85.30±0. 02	57.3±0. 08	10.80± 0.50
Root(200mg/ kg)ND	8.69±3. 24*	69.29±7. 60	72.69± 8.80	22.82±4. 81	92.10±0. 00	58.70± 0.02	7.50±6. 40
Root(400mg/ kg)ND	12.38± 4.28	72.23±1. 45*	78.62± 4.90	18.90±2. 42	88.20±0. 92	55.60± 1.90	7.78±0. 54

Values are expressed as Mean ± SD, n=6, *P<0.05 vs NC; a = P<0.05 vs DC

ND=non diabetic   NC =normal control, DC =Diabetic control

Discussion

In acute toxicity test, a total of 25 mice were used according to the method of Lorke (1983) for the determination of the dose extract that will produce the effect in 50% of the animal treated. At the end of the twenty four hours in each case, none of the doses gave a positive result and no ovate manifestation of toxicity was observed from 100mg/kg body weight up to a dose level of 5000mg/kg body weight.

Streptozotocin-induced diabetes is characterized by a severe loss in body weight (Al-Shamaorry et al., 1994), which might be the result of protein wasting due to unavailability of carbohydrate as an energy source (Chen and Ianuzzo, 1982). Tissue waisting is a characteristic of poor glycemic control in diabetes and this usually foster protein and fat mobilization (Cotran et al., 1999). Atangwho et al., 2007a and Okokon et al., 2011 reported significant weight reduction in untreated diabetic animals. This was also observed in the present study with untreated diabetes. The sustained but gradual reduction in weight of untreated diabetic rats over 28 days clearly indicated the deterioration in glucose control mechanism. This observation is a direct inverse of changes in blood glucose which rather increases over the period, hence establishing inverse relationship between blood glucose and weight changes in untreated diabetes. Treatment of diabetic rats with ethanolic root stem extract of V.amygdalina improved the

weight gain compared to untreated diabetic rats (Table 3a and 3b).The gradual appreciation in weight upon treatment indicates that the treatment would have allowed the tissues access to glucose, both to supply energy and build tissue material needed for growth (Atangwho *et al.*,2007a).

The cardinal body fluid parameter in diagnosis and progress monitoring, as a response to the treatment of diabetes mellitus has lesser blood glucose concentration (Loeb,1991).Fasting blood glucose at the end of 28 days treatment measured in the blood and serum of untreated diabetic rats was significantly higher (P<0.05) than non diabetic control by 3.1 and 6.4 folds respectively. Treatment caused significant decrease in serum glucose only with *V.amygdalina* stem (200 and 400) mg/kg b.w but a non significant reduction with *V.amygdalina* root extract (200mg/kg), which caused a significant reduction in treated non diabetic rats. The observed reduction in blood serum glucose of diabetics, is in accordance with Ebong *et al.*, (2008) earlier report on the *V.amygdalina* leaves. Some plants' extracts are reported to exert hypoglycaemic action by potentiating the insulin effect, either by increasing the pancreatic secretion of insulin from the cells of islets of langerhans or its release from bound insulin (Pari and Amarnah, 2004), while others act through extra pancreatic mechanisms by inhibition of hepatic glucose production (Edduoks *et al.*, 2003) or corrections of insulin resistance (Hu *et al.*, 2003). This stem extract may have acted through one of these mechanisms. The reduction in serum glucose of diabetics treated with stem extract relative to diabetic control probably may imply insulin mimetic action.

Enzymes appear frequently in blood as a result of cellular injury.The most widely used enzymes to assess hepatocellular injury are the aminotransferase (ALT and AST) whereas alkaline phosphatises are useful in diagnosis of hepatobilliary or cholestatic obstruction (Johnson and Fody, 1992).in this study, the damage caused by STZ was substantiated by the elevated level of AST, ALT and ALP in diabetic control rat. The increase might be due to leakages from the cytosol or changes in the permeability of the liver membrane. However treatment for 28days with stem and root extracts of *V.amygdalina* reduced the activity of this enzymes in a dose related manner compared with the diabetic control thus improving renal and hepatic functions. This observation concurred with report of Atangwho *et al.*, 2007c who reported the hepatoprotective effect of leaves extract of *V.amygdalina* in diabetic rat, its terpenoid extracts against carbon tetrachloride induced hepatotoxicity has equally been reported (Babalola *et al.*, 2001).

The data obtained from this study show significantly (p<0.05) decreased levels of total protein, albumin and increased total bilirubin in diabetic control group and this is consistent with the investigation when

compared to non diabetic control group (Tumevo *et al*., 2007) and Bakris, 2007). The administration of stem extract alone caused significant increased in total protein and albumin as compared with normal group which indicates stabilization of plasma membrane and protection of liver cell membrane.

Conclusion

The results of this study show that ethanolic stem and root extract of *Vernonia amygdalina* possess antidiabetic properties and liver function maintenance. This confirmation justifies its use in ethno medicine for the treatment of diabetes.

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