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Original Article

Improvement of glucose tolerance in rats fed with diets containing *Vernonia amygdalina* leaves

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ABSTRACT: The effect of 5% and 10% dietary incorporation of leaves of *Vernonia amygdalina* (VA) on oral glucose tolerance was studied in normoglycemic male albino rats. The feeding of the vegetable incorporated diets (5% *VA* and 10% *VA*) resulted in marked improvement in oral glucose tolerance in rats. After one week of diet administration, following an oral glucose load (3g/kg body weight), blood glucose concentration (BGC) (mg/dL) in rats fed the 5% *VA* peaked at 15minutes (147.24± 18.46), while the BGC in rats fed the control diet and 10% *VA* peaked at 30mins (180.00 ± 28.57 and 159.48 ± 16.07 respectively). After two weeks of feeding of the vegetable incorporated diets, post administration BGC peaked at 15 minutes in the test groups [5% VA diet (152.64± 33.46) and 10% VA diet group (121.95± 24.78)] while the peak remained at 30 minutes in the control group. After 3 weeks of diet administration BGC remained peaked at 15 minutes in the 5% and 10% VA diet groups (137.16 ± 61.29 and 132.75 ± 8.86 respectively). Rats in both test groups, after 3 weeks on the test diets, had their glycaemic indices reduced by 15-18%. These findings indicate that feeding on diets containing *Vernonia amygdalina* could positively modulate oral glucose tolerance. The vegetable could be useful in dietary management of conditions associated with oral glucose tolerance, and by extension, hyperglycemia.

KEYWORDS: Diabetes mellitus; Dietary incorporation; Oral glucose tolerance; *Vernonia amygdalina*

INTRODUCTION

The oral glucose tolerance test (OGTT) measures an individual organism's ability to utilize ingested glucose, the body's main source of energy, over a given period of time. OGTT is a good marker of the diabetic state and a test of immense value and when used in combination with fasting plasma glucose concentration, it facilitates the diagnosis of diabetes, as against using the latter alone. In fact, for a long time, it was the mainstay for the diagnosis of diabetes (Bartoli *et al.,* 2011).

Under normal physiological conditions, high blood glucose concentration promotes insulin release from the β-cells of the islets of Langerhans of the pancreas. Insulin stimulates the uptake of glucose by peripheral tissues especially skeletal muscle [by up-regulating the expression of glucose transporter-4 (GLUT-4) and by stimulating the exocytosis of stored GLUT-4], and promotes the storage of glucose in the liver (as glycogen) through the stimulation of glycogen synthase activity (Zunino, 2009; Zaid *et al.,* 2008). Loss of responsiveness to insulin by insulin-responsive tissues results in the sustained elevation of blood glucose concentration (hyperglycemia), and ultimately to type 2 diabetes mellitus, a metabolic condition that affects the metabolism of carbohydrates, lipids and proteins (Pareek *et al.,* 2009). Besides hyperglycemia, type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissues, and the eventual destruction of the β-cells of the islets of Langerhans (Guillausseau et al., 2008). It affects 285 million people (6.4%) globally, and is estimated to affect 438 million people by the year 2030, most of who would reside in developing countries (IDF, 2010). In fact, currently, diabetes

mellitus is thought to be the most prevalent endocrine disorder in Nigeria (Eseyin *et al.,* 2010).

A significant percentage of the population in sub-Saharan Africa and many countries in the developing and under-developed regions of the world live in rural communities and have limited access to conventional medical treatment. Conventionally the management of diabetes mellitus involves non-pharmacological (diet control and exercise) and pharmacological (administration of insulin and hypoglycaemic drugs) approaches. Administration of exogenous insulin and oral hypoglycaemic agents such as biguanides and sulfonyureas) are the available orthodox therapies for the management of diabetes. These agents however do not revert the course of diabetic complications and often come with some toxicity (Pareek *et al.,* 2009). The recommendation of the WHO supporting the search for plants that are effective in managing diabetes mellitus (WHO, 1980) and the thinking that botanicals are largely free from the toxicity associated with orthodox pharmaceuticals have emboldened the search for phytotherapeutics with anti-diabetic potentials. One plant that has been variously studied for usefulness in managing a wide array of medical conditions is *Vernonia amygdalina* Del.

Vernonia amygdalina Del (Asteraceae) is a perennial shrub that grows to 2-5 m in height, throughout tropical Africa, and is commonly called bitter leaf. Its leaves are used traditionally in the preparation of soups and porridges. The plant reportedly finds application in the management of a myriad of ailments (Ijeh and Ejike, 2011). The hypoglycemic potentials of *V. amygdalina* had been reported as early as two decades ago (Akah and Okafor, 1992; Ogbuokiri and Ekpechi, 1989). Other researchers have used different preparations/extracts of the plant leaves, and have reported significant blood glucose lowering properties of the plant (Ijeh and Ejike, 2011). In this report, we describe our investigation of the response of rats, fed a *V. amygdalina* incorporated diet, to an oral glucose challenge.

MATERIALS AND METHODS

Processing of plant materials and composition of test diets

Fresh leaves of *Vernonia amygdalina*. Del were harvested from a local farm in Owerri, Imo State, Nigeria and were botanically identified by Dr GGE Osuagwu of the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. The leaves were sorted to remove dead and wilted leaves and rinsed in clean tap water to remove dust and dirt. They were then air-dried at room temperature under a flowing fan until a constant dry weight was obtained. The leaves were milled to coarse fine powder, using a steel laboratory mill.

The test diet was compounded by mixing milled standard growers feed (Vital Feeds Nigeria Limited) with the dried milled *Vernonia amygdalina* leaves in ratios of 95:5 and 90:10 (feed/vegetable) to constitute the 5% VA and 10% VA diets respectively. The unmixed growers feed (100%) served as the control diet. The diets were then converted to pellets by extrusion through an improvised device made by neatly slicing the end of a 5 ml syringe.

Rats

Twenty four adult male Wistar rats (weight range 118–142 g) were obtained from the Animal Breeding Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. On arrival, the rats were acclimatized to the animal house for one week before being randomly (while controlling for weight) assigned to six plastic cages (2 cages for each group of eight rats). The rats were exposed to approximately 12 hour light/dark cycles under humid tropical conditions, and given tap water and feed *ad libitum* throughout the duration of the study.

Oral glucose tolerance test

Oral glucose tolerance test was carried out using a modification of the method described by Taiwo *et al.* (2009). Rats were fasted overnight and given an oral glucose load of 3 g/kg body weight *per os*. Following the oral glucose load, blood was obtained at 0, 15, 30, 45 and 60 minutes from the tail vein of the rat and analysed for glucose using a glucometer (Accu-check Advantage, Roche Diagnostics, Mannheim). The procedure was repeated after one, two and three weeks of feeding the test rats on the test diets. From the data generated from the OGTT, the positive incremental area under the curve (AUC) was calculated for each group using the equation:

$$AUC = \{[(t_1-t_0)\div 2] \times (C_0+C_1)\} + \{[(t_2-t_1)\div 2] \times (C_1+C_2)\} + \{[(t_3-t_2)\div 2] \times (C_3+C_2)\}\ldots$$

(where t = time and C = concentration of glucose) (Brouns *et al*., 2005).

The glycaemic index was calculated as:

$$GI = AUC_{test}/AUC_{control}$$

Statistical analysis

The data generated were subjected to descriptive statistical analysis, and a comparison of differences between means using One Way ANOVA. A significant threshold of $P < 0.05$ was employed for the analysis. Data analysis was carried out using SPSS version 18.0 (SPSS Inc, Chicago, IL). The results are presented as line graphs generated using Microsoft Excel (Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

The oral glucose tolerance test is a good and cheap measure of insulin secretion, sensitivity and glucose uptake (Rhee *et al*., 2010; Matsuda and DeFronzo, 1999) and since an impaired glucose tolerance is an indication of a deranged glucose metabolism and a pointer to subsequent diabetes

(Eseyin *et al.,* 2010), the test is good for studying agents that improve glucose tolerance and, by extension, are useful in the management of diabetes (and diabetic complications). Its main advantage is in its ability to detect stages of pre-diabetes more accurately than other methods, and its ability to investigate postprandial glucose levels in a physiological way (Luijf *et al.*, 2011).

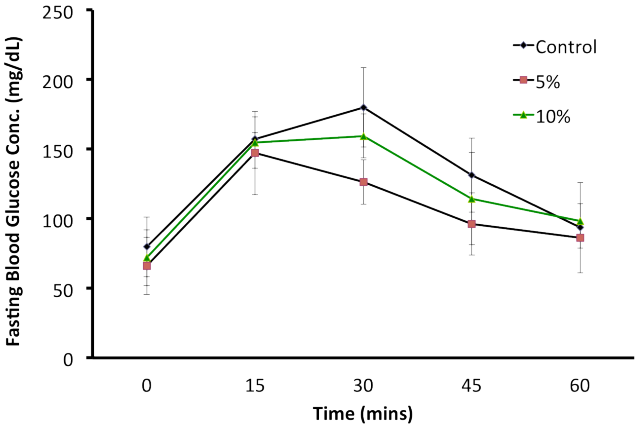

Figure 1: OGTT after one week of feeding on the 5 and 10% *Vernonia amygdalina* incorporated diets.

The results (Figures 1-3) revealed that after the first week of feeding on the test diets blood glucose concentration (BGC) peaked at 15 minutes in the group fed 5% *VA* incorporated diet while the group fed the control diet and the 10% *VA* diet had peak BGC at 30 minutes, following an oral glucose load. Blood glucose concentration however peaked at 15 minutes in the 5% *VA* diet and 10% *VA* diet groups after 3 weeks of feeding on the vegetable diet but the BGC peak remained at 30 minutes in the control group throughout the study. Also after two weeks of feeding on the test diet, BGC was significantly ($P < 0.05$) lower in the group fed 10% *VA* incorporated diet relative to the control group 30 minutes after the glucose load. After 3 weeks of feeding on the test diet there was a significant ($P < 0.05$) reduction in BGC at 30 minutes in both test groups, relative to the control group. Peak BGC was lower in the test groups throughout the period of the study indicating that *VA* had hypoglycaemic properties. Furthermore, by the end of the third week, both test diets considerably lowered the AUC's for the OGTT curves, giving glycaemic indices that had shrunk by more than 15% each (Table 1).

These findings show that dietary incorporation of *Vernonia amygdalina* improved oral glucose tolerance as indicated by the lower glucose concentrations at peak BGC in the test groups, the reduction in the time it took to reach the peak BGC and for the decline to begin, and the reduction in the AUC for the OGTT curves and the glycaemic indices of the test diets. There are two mechanisms that could explain the glycaemic effects observed here. The active principles in the test diets could (1) slow down glucose transport into the blood from the lumen of the gastro-intestinal tract, or (2) improve insulin secretion and/or sensitivity, and thus glucose transport from the blood to the other tissues, and its utilization there. Though these are speculations (as further studies are required to establish the exact mechanism(s) of action of the agents), it is obvious that agents that affect glucose metabolism employ the mentioned mechanisms. While the first mechanism ensures that glucose does not get over-loaded in the blood, the second ensures that the glucose is utilized rapidly by the cell (Kalsbeek *et al.*, 2010). The finding of lower glycaemic indices in the test groups at the end of the study supports the former mechanism, but does not preclude the latter.

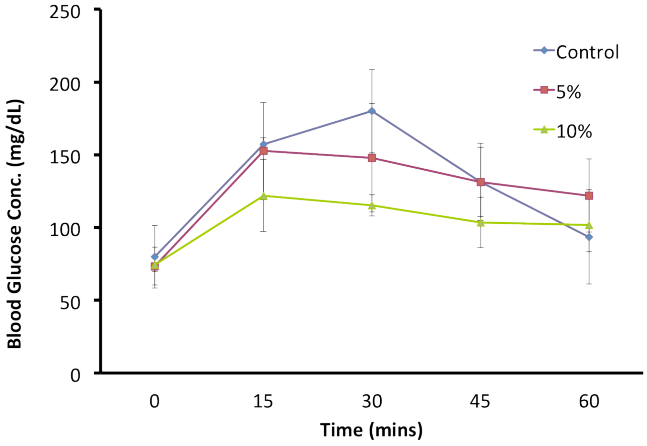

Figure 2: OGTT after two weeks of feeding on the 5 and 10% *Vernonia amygdalina* incorporated diets.

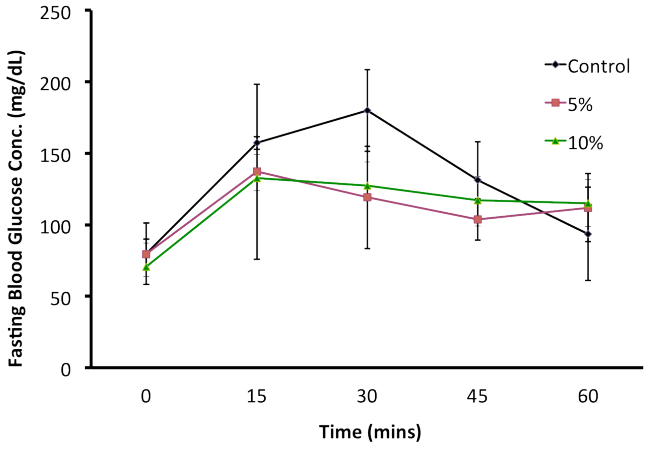

Figure 3: OGTT after three weeks of feeding on the 5 and 10% *Vernonia amygdalina* incorporated diets.

Table 1: Areas under the curve (AUC) for the effects of dietary incorporation of *Vernonia amygdalina* on the OGTT curves. VA represents *Vernonia amygdalina* incorporated diet.

	Week 1	Week 2	Week 3
	Incremental **areas under the OGTT curve**		
Control	8329.5	8329.5	8329.5
5% VA	6690.2	7944.9	6838.3
10% VA	7709.3	6429.8	7052.3
	Glycemic index (%)		
Control	1.00 (100)	1.00 (100)	1.00 (100)
5% VA	0.80 (80.3)	0.95 (95.4)	0.82 (82.1)
10% VA	0.93 (92.6)	0.77 (77.2)	0.85 (84.7)

These findings also show that the improvement in blood glucose tolerance was time dependent as seen in Figure 3 which shows that there was a decrease in peak blood glucose concentration after three weeks of feeding when compared with the peak observed after the first and second weeks of feeding the vegetable diet. This implies that the consumption of the test diet had a cumulative advantage. The improvement of glucose tolerance with time suggests that when consumed habitually, the test diet could be useful

in the prevention of diabetes; and for those already diabetic, it may be useful in regulating glucose utilization after a carbohydrate-rich meal. This is particularly useful, as the bulk of the challenge of diabetics is keeping the blood glucose levels within the physiological threshold. Pharmacologic agents used to achieve such regulation often result in hypoglycemia which could be very dangerous (Brod *et al*., 2011). Going by the data presented here, the consumption of *VA* as parts of the regular diet could be useful for diabetics as it could help regulate their blood glucose levels, thereby preventing hyperglycemia and in turn reduce their dependence on pharmacologic agents.

Several reports indicate that the extracts of *VA* have hypoglycaemic potentials (Ijeh and Ejike, 2011). However, little has been investigated about the use of diets incorporated with the plant leaves in the management of diabetes and related metabolic disorders. Because adherence to drug/dietary regimens by diabetic subjects is often poor (Davies *et al.,* 2013; Mann et al., 2009), the use of *VA* in diets may be very useful especially in the South Eastern parts of Nigeria where the plant leaves are used in the preparation of a variety of soups. This allows subjects take the preparations without the psychological feeling that they are taking medications, a factor that helps in ensuring compliance. Such use would maximize the utilization of all the pharmacologic principles in the plant leaves that might be important for the observed biochemical effects.

This study may be limited by its scope, as it did not investigate the mechanism of improvement in oral glucose tolerance, and the effects of the diet on metabolic parameters downstream of impaired glucose metabolism. Those are subjects of our on-going research. However, this is to our knowledge the first report of the usefulness of diets incorporated with the leaves of VA in the improvement of oral glucose tolerance. The inherent advantages of this approach are at the core of the strength of this study.

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Original Article

Evaluation of selected trace metals in some hypertensive subjects in a tertiary health institution in Southwest Nigeria.

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ABSTRACT: Published reports on the possible roles of trace metals in the aetiology of primary hypertension have not been consistent. This study investigated the possible aetiological role of zinc (Zn), manganese (Mn), copper (Cu) and selenium (Se) in primary hypertension. Atomic absorption spectrophotometry (AAS) was used to determine the serum levels of Zn, Cu, Mn and Se in 45 patients with primary hypertension (stage I and stage II) and 47 apparently healthy control subjects (normotensives and pre-hypertensives). Both patients and control subjects were classified based on the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7). The weight, height and blood pressure of all subjects were measured and their body mass indices (BMI) computed. The mean serum zinc concentration was significantly higher in the patients with hypertension than in the control subjects (135.78 ± 9.10 vs 130.80 ± 12.50 μg/ml, p = 0.032). However, serum levels of copper (68.16 ± 3.72 vs 68.53 ± 5.33 μg/dl, p = 0.697), manganese (63.11 ± 4.40 vs 62.87 ± 4.59 μg/dl, p = 0.800) and selenium (75.91 ± 5.66 vs 78.13 ± 5.92 μg/L, p = 0.070) were not statistically different between the patients and the control subjects. This study did not show any gender-, age- or obesity-related differences in serum level of zinc, copper, manganese and selenium. Elevated level of serum zinc may play an aetiological role in subjects with primary hypertension. However, further studies will be necessary to define the roles of trace elements in the aetiology of primary hypertension in these individuals.

KEYWORDS: hypertension; zinc; manganese; copper; selenium.

INTRODUCTION

Hypertension is one of the most prevalent diseases in developed and developing countries. The estimated total number of adults with hypertension in the year 2000 was 972 million: 333 million in economically developed countries, and 639 million in economically developing countries. This number is projected to increase by 60 % to a total of 1.56 billion in the year 2025. Most of the increase (i.e. a rise of 80 % from 639 million to 1.15 billion) will be in economically developing countries like Nigeria (Kearney *et al.*, 2005). Hypertension is an important modifiable risk factor for strokes, congestive heart failure, end-stage renal disease and vascular diseases (Flack *et al.*, 1995).

Hypertension, is defined in adults (≥18 years of age) as a systolic blood pressure of ≥ 140 mmHg or a diastolic blood pressure of ≥ 90 mmHg (Chobanian *et al.*, 2003; Mancia *et al.*, 2007). Hypertension can be primary or secondary. Primary (essential) hypertension refers to elevated blood pressure with no clear identifiable cause. Secondary hypertension on the other hand, refers to elevated blood pressure resulting from a specific and potentially treatable cause such as renal disease, endocrine causes or drugs

(Camm & Bunce, 2005). Though primary hypertension has no identifiable cause, there are known predisposing factors. The predisposing factors to primary hypertension include obesity, sedentary lifestyle, high salt intake, low calcium intake, low potassium intake, increasing age, low birth weight, familial predisposition, autonomic imbalance, and likely trace metals (Whelton, 1994).

Trace metals make up less than 0.01 per cent of the body's dry weight (Crook, 2006). They may be essential or non–essential micronutrients. Sources of trace metals include both plant and animal foods. Plant foods tend to be rich sources of trace elements such as copper and manganese while animal products provide most of the zinc in diets (Anderson & Zlotkin, 2010). A total of 28 elements have been documented to play a role in blood pressure control. However, the role of these elements in the aetiology and control of blood pressure has not been fully elucidated (Loyke, 2002). The individual elements react directly and indirectly in a variety of metabolic and structural activities known to participate in blood pressure regulation[9]. Contradictory results have been published about the relation between copper, selenium, zinc, manganese and blood pressure (Taittonen *et al*., 1997). Hypertension is the most common manifestation of hypercupremia. The World Health Organization (1974) warned that high levels of copper in the tissues are positively correlated with hypertension.

Melanin, the natural pigment found in the skin seems to be a factor in the aetiology of some forms of hypertension, most especially in the darker skinned population. The physical property of melanin causes it to bind to heavy metals (Larsson & Tjalve, 1978). A strong positive correlation between high serum copper and hypertension have been reported in the dark skinned population with the assumption that copper excess might be a strong factor in the aetiology of hypertension (Pfeiffer & Mailloux, 1987). An earlier study by Creason *et al.* (1976) found that blood copper level is significantly higher in hypertensive black military recruits as compared to white military recruits. Thus copper, through its ability to be sequestered by melanin and its strong correlation with hypertension, may well lie at the heart of the high prevalence of hypertension in black population. Copper depletion experiments with men and women have revealed increased blood pressure (Klevay, 2000).

Zinc deficiency may cause arteries to become hard, brittle and often inflamed instead of soft and flexible and this loss of flexibility is expected to raise the blood pressure, in particular the systolic pressure. Zinc has been reported to lower blood pressure in some hypertensive patients (Pfeiffer & Lamola, 1983). The study also observed that oral doses of manganese causes a rise in blood pressure in patients over 40 years of age. Some other reports have shown that deficiency of Cu, Se, Zn and Mn might be associated with an increased risk of hypertension (Russo *et al*., 1998; Hajjar & Kotchen, 2003). On the other hand, Taneja and Mandal (2007) demonstrated high level of zinc in some hypertensives. However, another study (Ekmekci *et al*., 2003) did not observe any significant difference between serum zinc level of subjects with essential hypertension and normal controls.

Our literature search shows that there is a dearth of published reports on the relationship between trace elements and blood pressure in Nigeria and Africa in general. In this study, we report our study of the level of trace elements in normal subjects without hypertension and patients with newly diagnosed hypertension, and assessed the possible causal role of trace metals in the aetiology of hypertension in Nigerian Africans. The objectives of this study were to (1) determine the level of copper, selenium, zinc and manganese in the serum of patients with primary hypertension and subjects without hypertension; and (2) to determine if there is a relationship between the concentration of these trace metals and the level of blood pressure in patients with hypertension.

MATERIALS AND METHODS

Study Location

This study was carried out in Osogbo, a city with a population of approximately 450,000 people and located in the heart of South Western Nigeria.

Subjects

A total of ninety–two (92) subjects participated in the study. The subjects were grouped into two categories comprising of forty five (45) patients with primary hypertension attending the medical clinic of the Ladoke Akintola University Teaching Hospital, Osogbo and forty–seven (47) control subjects. The age ranged between 30 to 74 years. Informed consent was obtained from each of the subjects after the study was explained to them. All the control subjects had normal blood pressure according to the JNC 7 classification of hypertension. Excluded from the study were subjects that have glycosuria, are less than 30 years and those that had abnormal urinalysis or urinary sediments that may suggest the presence of kidney disease. Test subjects with newly diagnosed hypertension, not known to be on any anti-hypertensive drugs and who were not on any special diets were selected for the study.

Data were collected on each patient using a structured questionnaire. Body mass index (BMI) was calculated using the formula: weight/height^2 (kg/m^2). All patients were screened for glycosuria, and those with a history of other illnesses other than hypertension were excluded from the study.

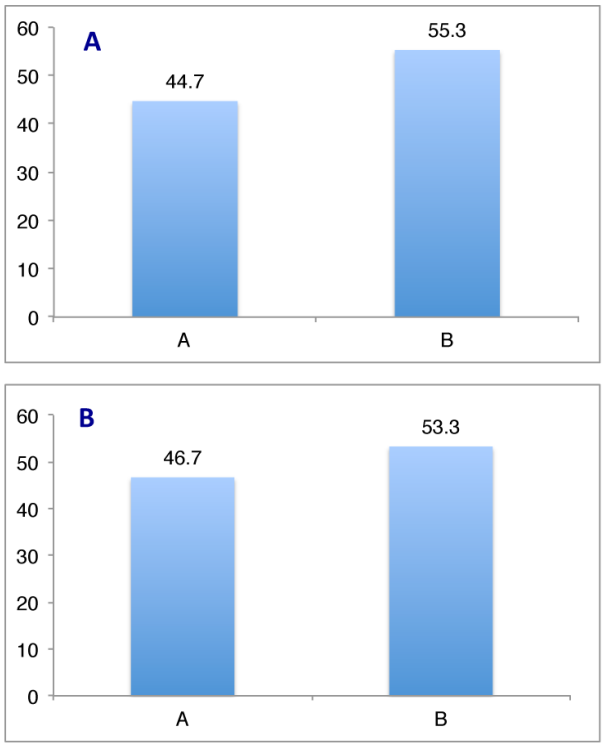

Figure 1: (A) Control subjects classified according to the JNC 7 report. A = < 120 / 80 mm Hg; B = ≥ 120 / 80 mm Hg but < 140 / 90 mm Hg (B) Patients with primary hypertension classified according to the JNC 7 report A = ≥ 140 / 90 mm Hg but < 160 / 100 mm Hg; B = ≥ 160 / 100 mm Hg

Table 1: Statistical comparison of baseline demographic and clinical characteristics between patients and control subjects.

CHARACTERISTICS	PATIENTS N = 45 (Mean ± SD)	CONTROLS N = 47 (Mean ± SD)	p – value
Age (years)	50.98 ± 10.37	42.28 ± 8.12	0.001*
Weight (kg)	73.47 ± 15.55	73.28 ± 15.19	0.953**
Height (m)	1.66 ± 0.11	1.76 ± 0.09	0.043*
BMI (kg/m^2)	26.75 ± 5.48	25.38 ± 5.51	0.238**
SBP (mmHg)	151.99 ± 18.72	116.60 ± 9.79	0.001*
DBP (mmHg)	97.62 ± 10.62	77.01 ± 7.52	0.001*

BMI = Body Mass Index; SBP = systolic blood pressure;
DBP = diastolic blood pressure; ** = not significant; * = significant

Blood pressure was taken using a validated, digital automatic blood pressure monitor A&D UA–767 (Rogoza *et al.*, 2000) after the patient had rested for five minutes and after ensuring that he or she was not on any anti–hypertensive drug or alcohol or coffee within 30 minutes of measuring the blood pressure. Three blood pressure readings were taken at intervals of two minutes for each clinic visit, and the average of the last two readings was calculated. The diagnosis of hypertension was made when the average diastolic blood pressure ≥90 mmHg or when the average systolic blood pressure is ≥140 mmHg on at least two occasions (JNC VI). 5ml of venous blood was drawn from each subject and control into plain serum bottles. The blood sample was centrifuged at 4000rpm for 15 minutes at room temperature to separate the serum. The serum was dispensed into another plain bottle and stored at -20^{0}C until analysis. The serum concentration of copper, manganese, zinc and selenium were determined with flame atomic absorption spectrophotometer using a direct method as described by Kaneko (1999). The measurement was performed on a Beck (200) atomic absorption spectrophotometer.

Statistical Analysis

The SPSS software package was used for statistical analysis and graphical representation by Microsoft excel software. Continuous variables were summarized and displayed as means ± standard deviation (SD). Differences between continuous variables were assessed using ANOVA (analysis of variance). Comparison of means was done using the student's t–test. Pearson correlation coefficient was used to measure the level of association between variables. A p value of < 0.05 was considered to be statistically significant.

RESULTS

Baseline Demographic and Clinical Characteristics

The comparison of the baseline demographic and clinical characteristics in subjects indicates that the mean age, systolic and diastolic blood pressures were significantly higher in patients than in controls. The BMI was not significantly altered (Table 1)

Table 2: Statistical comparison of biochemical parameters between the patients and control subjects.

BIOCHEMICAL PARAMETERS	PATIENTS N = 45 (Mean ± SD)	CONTROLS N = 47 (Mean ± SD)	P – value
Zn (μg/ml)	135.78 ± 9.10	130.80 ± 12.50	0.032*
Cu (μg/dl)	68.16 ± 3.72	68.53 ± 5.33	0.697**
Mn (μg/dl)	63.11 ± 4.40	62.87 ± 4.59	0.800**
Se (μg/L)	75.91 ± 5.66	78.13 ± 5.92	0.070**

* = significant; ** = not significant

Table 3: Comparison between blood pressure and biochemical parameters of the study population with normal blood pressure as compared with those with pre-hypertension, stage i and stage ii hypertension

BIOCHEMICAL PARAMETERS	NORMOTENSIVE N = 21 MEAN ± SD	PRE-HYPERTENSION N = 26 MEAN ± SD	p^a	STAGE I N = 21 MEAN ± SD	p^b	STAGE II N = 24 MEAN ± SD	p^c	p^d	p^e	p^f
Zn (µg/ml)	129.81 ± 13.17	133.27 ± 9.43	0.249**	133.29 ± 8.05	0.289**	141.33 ± 11.84	0.012*	0.994**	0.025*	0.027*
Cu (µg/dl)	69.07 ± 5.53	67.93 ± 4.62	0.395**	67.29 ± 3.68	0.205**	69.50 ± 3.23	0.803**	0.596**	0.291**	0.093**
Mn (µg/dl)	62.14 ± 4.85	64.20 ± 3.85	0.075**	63.86 ± 4.41	0.205**	60.50 ± 4.15	0.312**	0.769**	0.009*	0.040*
Se (µg/L)	78.24 ± 6.08	76.90 ± 5.57	0.380**	76.62 ± 5.92	0.351**	75.25 ± 6.14	0.161**	0.864**	0.405**	0.533**

p^a = p-value for comparison between study participants with normal blood pressure and those with pre-hypertension, p^b = p-value for comparison between study participants with normal blood pressure and those with stage I hypertension, p^c = p-value for comparison between study participants with normal blood pressure and those with stage II hypertension, p^d = p-value for comparison between study participants with pre-hypertension and those with stage I hypertension, p^e = p-value for comparism between study participants with pre-hypertension and those with stage II, p^f = p-value for comparison between study participants with stage I hypertension and those with stage II hypertension, * = significant, ** = not significant.

Among the controls, 21 participants (44.7%) had blood pressure of less than 120/80 mmHg while 26 participants (55.3%) had blood pressure of between ≥120/80 mmHg and <140/90 mmHg (Fig 1A). 21 (46.7%) of patients reported a blood pressure of between ≥140/90 mmHg to <160/100 mmHg while 24 patients (53.3%) had a BP of ≥160/100 mmHg (Figure 1B)

Biochemical Parameters

Patients with hypertension had significantly higher serum zinc than control subjects ($p<0.05$). Mean serum copper, magnesium and selenium did not differ significantly between patients and controls (Table 2). It was also observed that serum zinc increased progressively from those with normal BP to stage II hypertensives. Significant differences were particularly seen when those with normal BP were compared with stage II hypertensives ($p<0.05$) and between stage I and stage II hypertensives ($p<0.05$) (Table 3). There were also significant differences between the mean serum manganese of subjects with pre-hypertension and those with stage II hypertension ($p < 0.01$), and serum manganese of subjects with stage I hypertension and those with stage II hypertension ($p < 0.05$). Comparison of biochemical parameters in both the control and patient groups with different classes of BMI (Tables 4 & Table 5) did not reveal any significant relationship between the level of trace metals across the categories of BMI in individual groups.

DISCUSSION

This study showed that the mean serum levels of zinc in subjects with hypertension was significantly higher than that in the control subjects. This seemed to increase progressively from prehypertensives to those with stage II hypertension. The result is consistent with that obtained by Henrotte *et al.* (1990) and Davydenko *et al.* (1995) who found a significant association between high level of zinc in serum and primary hypertension. Excess serum level of zinc is thought to cause an increase in intracellular zinc within the cell which in turn causes a rise of free calcium ion level in the smooth muscular layer of blood vessels, with consequent

vasoconstriction, increased peripheral resistance and elevated blood pressure. Tubek (2005) and Tomat *et al.* (2005) on the other hand found decreased level of serum zinc in hypertension when compared to controls. These authors showed that deficiency in serum zinc cause diminished nitric oxide activity with consequent elevation of blood pressure. The findings of Tubek (2005) and Tomat *et al.* (2005) also suggest however that zinc deficiency may be involved in the aetiology of primary hypertension which is contradictory to the finding of the present study. It appears that the severity of hypertension affects zinc levels or vice versa and this might in part explain this contradiction. In addition, Tubek (2005) used recently weaned animal models but Tomat *et al.* (2005) also demonstrated that zinc efflux

Table 4: Comparison of biochemical parameters between classes of BMI of control subjects

BIOCHEMICAL PARAMETERS	BMI 1 = 28 2 = 10 3 = 9	CONTROLS N = 47 Mean ± SD	p^a	p^b	p^c
	1	130.34 ± 12.49			
Zn (μg/ml)	2	133.90 ± 15.91	0.727**	0.476**	0.399**
	3	128.99 ± 8.34			
	1	63.46 ± 4.63			
Mn (μg/dl)	2	60.80 ± 4.57	0.941**	0.126**	0.231**
	3	63.33 ± 4.30			
	1	77.21 ± 5.20			
Se (μg/L)	2	79.60 ± 7.88	0.306**	0.286**	0.934**
	3	79.33 ± 5.72			
	1	69.57 ± 4.85			
Cu (μg/dl)	2	67.30 ± 6.24	0.140**	0.246**	0.819**
	3	66.67 ± 5.55			

BMI = body mass index, ** = not significant, **1** = (15 - 24) kg/m^2, **2** = (25 - 29) kg/m^2, **3** = (30 - 50) kg/m^2, **Zn** = zinc, **Mn** = manganese, **Cu** = copper, **Se** = selenium, p^a = p-value for comparison between study participants with normal BMI and those that are obese, p^b = p-value for comparison between study participants with normal BMI and those that are over-weight, p^c = p-value for comparison between study participants that are over-weight and those with obesity, **N** = total number of patients/subjects.

Table 5: Comparison of biochemical parameters between classes of BMI of patients

BIOCHEMICAL PARAMETERS	BMI 1 = 28 2 = 15 3 = 13	PATIENTS N = 45 Mean ± SD	p^a	p^b	p^c
	1	136.71 ± 9.76			
Zn (μg/ml)	2	133.67 ± 5.27	0.327**	0.291**	0.941**
	3	137.00 ± 11.64			
	1	63.71 ± 5.10			
Mn (μg/dl)	2	62.33 ± 3.85	0.561**	0.402**	0.787**
	3	63.23 ± 4.21			
	1	75.12 ± 4.95			
Se (μg/L)	2	77.67 ± 7.16	0.324**	0.246**	0.912**
	3	74.92 ± 4.41			
	1	67.76 ± 3.98			
Cu (μg/dl)	2	67.73 ± 4.28	0.308**	0.983**	0.285**
	3	69.15 ± 4.28			

BMI = body mass index, ** = not significant, **1** = (15 - 24) kg/m^2, **2** = (25 - 29) kg/m^2, **3** = (30 – 50) kg/m^2, **Zn** = zinc, **Mn** = manganese, **Cu** = copper, **Se** = selenium, p^c = p-value for comparison between study participants with normal BMI and those that are obese, p^b = p-value for comparison between study participants with normal BMI and those that are over-weight, p^a = p-value for comparison between study participants that are over-weight and those with obesity, **N** =total number of patients/subjects.

rate from lymphocytes decreased with severe hypertension but increased with mild hypertension. From the foregoing, the role of zinc in the aetiology of primary hypertension appears yet to be fully established. However, This study did not find any significant statistical difference in serum zinc, selenium, manganese and copper in relation to obesity (BMI). Although Ghayour-Mobarhan *et al.* (2005) did not find any significant difference between obese and non-obese subjects with regard to selenium which is similar to the findings of this study, Tungtrongchitr *et al.* (2003) however found lower serum zinc levels and higher serum copper in obese compared with non-obese subjects while Taneja *et al.* (1996) found significantly higher serum zinc levels in obese subjects compared to control group. Manganese intake has been associated with an increased risk of hypertension in a representative sample of the adult Korean population (Lee & Kim, 2011). This study revealed that the serum levels of manganese in the patients with hypertension was higher than that of the control subjects but the difference was not statistically significant. However, a relationship between those with stage 1 and stage 2 hypertension, with regards to manganese was observed. Significant lower manganese level was seen in stage 2 hypertensives compared to stage 1 patients. Overall, there was no consistency in manganese level across the various stages of hypertension in this study. Even though it has been reported that copper deficiency is associated with impaired endothelium-dependent arterial relaxation that may result in hypertension causing extensive damage to arteries (Fell *et al.*, 1980), there was no statistically significant change in the plasma copper level of hypertensive subjects in the present study.

In conclusion, this study showed that serum levels of zinc are significantly higher in patients with primary hypertension. There were insignificant changes in the concentrations of maganese, copper and selenium in patients with hypertension compared to subjects without hypertension. In addition, there were no obesity-related differences in serum zinc, copper, manganese and selenium levels. These findings show that it is important to continue with further research to elucidate the possible roles of these trace elements in the aetiology of primary hypertension.

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Original Article

Extracellular release of acid phosphatase from blood stream forms of *Trypanosoma brucei brucei.*

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ABSTRACT: Acid phosphatase (ACP) activity was demonstrated in blood stream form of *Trypanosome brucei brucei* harvested from infected Wister rats by Ion Exchange DEAE Cellulose 52 chromatography. Whole parasite extract (WPE) and Excretory Secretory Extract (ESE) were prepared and analyzed for acid phosphatase activity. A higher ACP activity (85.5 μmol/min) was recorded in WPE compared to ESE (36.8 μmol/min). ACP activity in ESE is suggestive of the presence of a cell rich enzyme. Phase separation of the extracts using the detergent Triton X-114 (TX-114), resulted in protein partitioning into aqueous and detergent phases. ACP activity was higher in the detergent phases (56.2 μmol/min and 28.8 μmol/min) of WPE and ESE respectively. ACP activity recorded in the aqueous phases of WPE and EPE was 27.8 and 7.6 μmol/min respectively. On a Size Exclusion chromatography column using Sephacryl-300, ESE emerged as five distinct protein peaks. ACP activity of the eluted fractions showed two peaks of relative molecular weights 195 and 325 KD. This study shows that T. brucei releases acid phosphatase extracellularly via a yet to be determined mechanism. Acid phosphatase activity in ESE is indicative of a soluble enzyme within the cell matrix which may also play an important role in the pathology of African Trypanosomiasis.

KEYWORDS: Trypanosoma brucei; acid phosphatase; enzyme secretion.

INTRODUCTION

African trypanosomes are protozoan parasites of man and animals. Several species of trypanosomes infect mammals with the three most important belonging to the *Trypanosoma brucei* complex. *T. bucei brucei* causes “nagana” in cattle, *T. brucei gambiense* causes chronic human disease in Western and Central Africa and *T brucei rhodesiense* results in acute human infections in Eastern Africa (Barrett, 1999). The disease in humans is termed the “Human African trypanosomiasis” (sleeping sickness). The transmission of the parasite and resulting disease in cattle renders a large area of sub Saharan Africa refractory to the production of cattle; causing nutrient deficiencies and significant economic losses in livestock. Trypanosomes cause devastating diseases resulting in high human mortality; sleeping sickness is invariably fatal if not treated (Kennedy, 2004).

Several enzymes, including acid phosphatase, acid pyrophosphate and adenylate cyclase have been identified in the flagellar pocket membrane of *Trypanosoma brucei* (Walter & Opperdoes, 1982). Acid phosphatases are a group of enzymes found in many plant and animal species (Bull *et al.*, 2004). They have the ability to catalyse the hydrolysis of orthophosphate monoesters under acidic conditions and are known to facilitate important physiological changes within cells (Bull *et al.*, 2004). Whether the enzyme is of plant, animal or microbial origin, acid phosphatase from these widely diverse origins can be divided into two main classes; high and low molecular weight enzymes (Tanizaki *et al.*, 1977), which hydrolyses orthophosphoric monoesters at acid pH. These two classes differ with respect to their intracellular distribution, substrate specificity, behaviour towards inhibitors, sequence length, amino acid homology, tissues and chromosomal origin (Bull *et al.*, 2004; Igarashi & Hollander, 1968; Chaimovich, & Nome, 1970). Acid

phosphatases have been identified in parasitic protozoa of the family *Trypanostomastidae,* including *Crithidia* species (McLauqhlin *et al*., 1976) salivarian Trypanosomes (Brooker & Vickerman, 1968) and *Trypanosoma cruzi* (Avila *et al*., 1979; Steiger *et al*., 1979). This enzyme has been cytochemically localized within these flagellates to the flagella pocket and vesicles adjacent to the base of the flagellum, endosome, lysosomal compartments and within reservoir surrounding emerging flagellum of Trypanosomes (Langreth & Balber, 1975; Schell *et al*., 1990). There are reports of at least two different acid phosphatases (Langreth & Balber, 1975; Schell *et al*., 1990) in *Trypanosoma brucei* and three acid phosphatases (Amlabu *et al*., 2009) from the lysosome of blood stream forms of *Trypanosoma brucei brucei.* The presence of a membrane extracellular glycosylated acid phosphatase in *Leishmania donovani* suggested that these parasites secrete acid phosphatase into the surrounding medium (Menz *et al*., 1991; Cazzulo *et al*., 1990). Acid phosphatases are proposed to be involved in the regulation of pyridoxal phosphate requiring enzymes, in steroid transport, vitamin B6 metabolism and in lipid metabolism (Andrews & Turner, 1996; Kaplow & Burstone, 1964; Blank & Snyder, 1970). There are several possibilities to the physiological role of the major bound acid phosphatase. Some ACP located on the cell surface catalyses the hydrolysis of key glycolytic intermediate Fructose–1,6–phosphate and thus raises the possibility that once the parasite has been internalized by the host, the parasite derived phosphatase activity could disturb glycolysis specifically and energy metabolism in general. The parasite ACP while acting together with its proteases could cause tissue degradation thus easing tissue invasion, including the blood-brain barrier, by the trypanosome (Lonsdale-Eccles & Grab, 2002). ACP has also been proposed as one of the virulence factors in Leishmanaiasis (Shakarian & Dwyer, 2000; Love *et al*., 1998).

The flagellar pocket is the sole site of endocytosis and secretion in trypanosomes and several studies have suggested the presence of membrane bound ACP in *T. brucei*. This necessitates the investigation of the presence of secreted ACP from blood stream forms of trypanosomes and the likely role of such exocytosed molecules in the pathology of African Trypanosomiasis. This paper reports the acid phosphatase activity in the excretory secretory extract of *T. brucei brucei.*

MATERIALS AND METHODS

Parasite maintenance and harvest

Trypanosoma brucei brucei were collected from the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Nigeria and maintained in the laboratory in Albino rats in house breed at the Department of Zoology, University of Ibadan. Age and sex matched rats were employed in this study. The rats (10) were each inoculated intraperitoneally with 10^4 *T. b. brucei in* 0.5 ml of PSG. At peak parasitaemia ($1xl0^9$/ml), Trypanosomes were harvested from the rats by cardiac puncture using tri-Sodium citrate as anticoagulant. The parasites were separated from blood by DEAE-Cellulose ion exchange chromatography, using Phosphate Saline Glucose (PSG) pH 8.0 as column buffer (Lanhan & Godfrey, 1970).

Whole Parasite Extract (WPE) and Excretory-Secretory Extract (ESE)

Pelleted trypanosomes obtained by centrifugating eluent from DEAE Cellulose Chromatography were suspended in phosphate saline glucose buffer (PSG) pH 8.0. Trypanosomes were counted on a haemocytometer and $1x10^8$/ml was incubated in PSG at 4 °C for 30 minutes and then centrifuged at 1000 g for 10 minutes in a Beckman centrifuge (J-21). The resulting supernatant (ESE) was decanted and concentrated at 7500 g for 45minutes using Centricon-10 (Millipore). The protein content of ESE collected was determined as described by Bradford using BSA as standard (Bradford, 1976).

To prepare the whole parasite extract, pelleted trypanosomes separated from blood by DEAE-Cellulose Chromatography were lysed using silicon carbide. Lysate was re-suspended in PSG and centrifuged at 1000 g for 10 minutes to remove silicon carbide. Supernatant lysate was incubated for 1 hour at 4 °C concentrated using Centricon 10. Acid phosphatase activity was determined for both WPE and ESE extracts.

Triton X-114 Phase Separation.

WPE and ESE were subjected to Triton-X 114 treatment by placing a mixture of 100μ1 each of test samples, 367 μl of l0 mM Tris-HCl, 150 mM NaCl buffer and 1% Triton X-114, which had been maintained at 4 °C onto a 600 μl of 0.06% Sucrose cushion. The mixture was incubated at 30°C until cloudy and centrifuged at 800 g for 5 minutes. Clearly defined detergent and aqueous phases were formed, which were aspirated into separate tubes and the sucrose cushion was discarded. Acid phosphatase activity was determined for each phase.

Determination of Acid Phosphatase Activity

Acid phosphatase activity was determined as described by Steiger *et al.* (1979). Briefly, to 1 ml of sample was added 1 ml of freshly prepared 0.1 M 4-Methylumbeilliferly phosphate in 0.05 M Sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 10 minutes, enzyme reaction was terminated by addition 3 ml of 0.05 M Glycine-Sodium hydroxide buffer (pH 10.4). A reaction mixture in which substrate was incubated in the absence of test sample (enzyme) and the appropriate volume of enzyme added after the addition of glycine–sodium hydroxide buffer, compensating for non-enzymatic hydrolysis, served as control. A blank mixture was prepared containing the substrate and buffer in the absence of the enzyme. Enzyme activity (RF values) was determined on a spectroflurometer using 4-Methylumbelliferon as standard.

The standard was prepared by dissolving one μmol/ml of 4-Methylumbelliferon in 0.05M Sodium Acetate-Acetic Acid buffer, pH 5.0. Serial dilutions (0.0-0.2 μmol/ml) were prepared. To each dilution was added 3ml Glycine-Sodium hydroxide buffer pH 10.4 and mixed thoroughly. The Relative Fluorescence (RF) was determined for each dilution at Excitation (EX) = 364 nm, Emission (EM) = 448 nm at 50% calibration. The RF values obtained were plotted on a linear graph against sample concentration.

Enzyme activity (μm MUP) for each fraction collected was calculated from RF values;

$$\mu\text{m MUP} = \frac{standard\ MUP}{50\%\ calibration} xRF\ .$$

The specific activity of acid phosphatase was calculated using the formula:

μm MUP/mg Protein =

$$\frac{\frac{1000}{vol\ of\ sample} X \frac{\mu mMUP}{1}}{mgP} \quad \frac{\frac{1000}{\text{volofsample}} X \frac{\mu\text{mMUP}}{1}}{\text{mgP}}$$

Size Exclusion Chromatography

Partial purification of ESE was carried out by gel fitration on a pre-swollen Sephacryl-300 (BIO-RAD) column and equilibrated according to manufacturer instructions with 0.05 M Sodium acetate acetic acid buffer (pH 5). A 2 ml sample was layered on packed column and eluted with the same buffer at 4 °C at a flow rate of 18 ml/cm^2-hr. The eluents were collected in 3ml fractions and absorbance of each fraction was measured at 280 nm to locate protein containing fractions. The molecular weight of each protein peak obtained was estimated after the calibration of the column and acid phosphatase activity was determined for each fraction collected and fractions with activity were pooled for electrophoresis.

Column Void volume

This was determined using Blue dextran 2000 and the elution volume (Vo) of Blue dextran 2000 is equal to column void volume. A fresh solution of Blue Dextran 2000 (1 mg/ml) in eluent buffer was prepared and layered on gel in column after elution of ESE. Blue Dextran was eluted at 18 ml/cm^2-hr in 3 ml fractions. Absorbance of the eluents was monitored at A_{280}. The fraction with highest A^o value corresponds to Vo of the column.

Column Calibration and Molecular Weight Estimation

Standard proteins (Aldolase 5 mg/ml, Ferritin 1 mg/ml, and Bovine Serum Albumin 5 mg/ml) of known molecular weights were used for calibration by dissolving each protein in the eluent buffer and allowed to stand at room temperature for 10 minutes as recommended by (Pharmacia) the manufacturer. A 1 ml of the dissolved proteins was layered on the column and eluted at 18 ml/cm^2-hr and collected in 3 ml fractions. Vo for each protein was measured at A_{280}. The molecular weights of these standard proteins were: Aldolase 158 kD, Ferritin 440 kD, Bovine serum albumin (BSA) 58 kD.The relative molecular weight (K_{av}) of the eluted proteins were calculated.

$$\text{Kav} = \frac{Ve - Vo}{Vt - Vo}$$

$\frac{Ve}{Vo}$ = relative mobility

where Ve = Elution volume ,

Vt = Total bed volume,

Vo = Column void volume

The K_{av} values for each standard protein was plotted on a linear scale against their corresponding molecular weights and the points on the calibration curve which corresponds to the calculated K_{av} values for the protein peaks identified in ESE was equivalent to the estimated molecular weight of the peak.

RESULTS AND DISCUSSION

In the acid phosphatase assay of ESE and WPE, enzyme activity was recorded in both the WPE (85.8 μmol/min) and ESE (36.8 μmol/min). The specific activity of acid phosphatase recorded was 99.88 μmol/mgprotein/min and 46.82 μmol/mg protein/min for ESE and WPE respectively (Table 1).

When the extracts were subjected to phase separation with TX-114, ACP activity was recorded in the aqueous and detergent phases of both extracts. The enzyme activity recorded was higher in the detergent phase of both ESE and WPE compared with aqueous phases. The enzyme activity in both phases of WPE was 97.9% of total activity in extract before Tx-114 treatment while acid phosphatases activity recorded in ESE after treatment with TX-11 was 99% of untreated extract (Table 2).

The absorbance profile (A_{280}) of protein fractions obtained from size exclusion chromatography (Figure 1) showed five protein peaks of relative molecular weights 31, 50, 72, 160 and 298 kD. Acid phosphatase activity of the protein fractions showing 2 peaks of relative molecular weight 195 and 325 kD is shown in Figure 1. The column void volume was 80 ml.

The possible physiological function for the extracellular acid phosphatase in *Trypanosoma brucei* is yet unknown. It may allow the intake of necessary orthophosphate from the organic phosphates in the blood circulation. In this study acid phosphatase activity in ESE is suggestive of extracellular release into the incubation medium indicating the presence of a cell rich acid phosphatase. This is in consonance with the reports of Brooker (1971) which proposed that the acid phosphatase activity observed in the flagellar pocket reservoir was as a result of exocytotic defecation processes. It was however reported that ACP in *T. congolense* did not appear to be secreted into the surrounding medium by living parasites (Tosomba *et al.*, 1996).

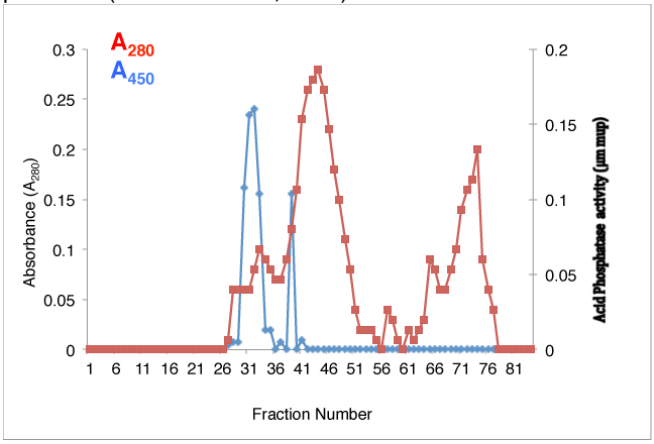

Figure 1: Protein profile and Acid phosphates activity in eluted fractions from column chromatography of excretory secretory extract of *Trypanosoma brucei* blood stream forms.

Table 1: Acid Phosphatase activity in extracts from *Trypanosoma brucei brucei*

	Enzyme Activity (μmol/min)*	Specific Activity (μmol/mgProtein/min)	Protein content (mg/ml)
Whole Parasite Extract (WPE)	85.8	99.88	0.859
Excretory Secretory Extract (ESE)	36.8	46.82	0.786

**μmol/min is amount of substrate hydrolyzed per minute*

Table 2: Acid Phosphatse activity in aqueous and detergent phases of TX-114 treated extracts of *Trypanosoma brucei brucei*

	Relative Fluorescence (RF)	Enzyme Activity (μmol/min)	Specific Activity (μmol/mgProtein/min)	% yield
Whole Parasite Extract (WPE)	21.45	85.8	99.88	-
Aqueous phase	6.95	27.8	32.36	32.4
Detergent phase	14.05	56.2	65.42	65.5
Excretory Secretory Extract (ESE)	9.2	36.8	46.82	-
Aqueous phase	1.9	7.6	9.67	20.7
Detergent phase	7.2	28.8	36.64	78.3

The acid phosphatase activity recorded in WPE suggests the presence of membrane bound acid phosphatase which are released after fractionation of the parasite membrane. Williamson & McLauren (1981) suggested that ACP in *Trypanosoma rhodesiense* was membrane bound while Zingales *et al.* (1979) reported an enrichment of acid phosphatase activity in the plasma membrane fractions and there were suggestions that portions of these enzymes make up constituents of plasma membrane of *Leptoma collosoma* and *Leishmania donovani* (Gottleb and Dwyer, 1981). Observations from this study suggest the presence of two acid phosphatase types, a membrane bound and a cytosolic form. There were reports of a membrane and an extracellularly glycosylated acid phosphatase in *L donvani* (Menz *et al.*, 1991). Dietma Schnell *et al.* (1990) also reported the presence of two different acid phosphatases in *T brucei.* They further suggested that acid phosphatase activity appeared to be confined to the intracellular compartments, which directly communicate with the flagellar pocket.

The higher enzyme activity recorded in the detergent fractions of the extract after Tx-114 treatment is in consonance with Langreth & Balber (1975), who had found that more than half of the acid phosphatase activity of trypanosome blood stream forms was not detected unless activated in some way by a detergent. The presence, in this study, of enzyme activity in the hydrophobic aqueous phase is suggestive of a soluble enzyme. This could be attributed to the diffusion of the enzyme along a gradient resulting in extra-cellular release of acid phosphatase. Eeckhont (1972) had reported the accessibility of the enzyme to the exterior through a diffusion barrier across the endoplasmic reticulum in cells.

The eluted protein fractions had acid phosphatase peaks of molecular weight 195 and 325 kD, which could suggest that the enzyme may be made up of subunits similar to those reported by Menz *et al.* (1991) in which the presence of dimeric units of acid phosphatases in *L donovani* were suggested. Allen *et al.* (1984) reported a soluble 128 Kd acid phosphatase composed of 65,000 and 68,000 subunits.

Contrary to the report of Tosomba *et al.* (1996) that acid phosphatase did not appear to be secreted into the surrounding medium by living parasites, this study has shown a high enzyme activity in the medium (ESE) in which live parasites where incubated. Thus it was shown in this study that *T brucei* releases acid phosphatase extracellularly although the mechanisms, pathway and its role in the pathology of trypanosomiasis remains to be thoroughly investigated.

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Original Article

Hepatotoxicological evaluation of water-soluble fraction (WSF) of Bonny Light crude oil (BLCO) in Wistar albino rats

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ABSTRACT: Background: It is common practice to pay more attention to the clearing of visible surface petroleum spills in streams and rivers which serve as the main source of drinking water in polluted sites in the Niger Delta area rather than taking cognizance of dissolved aromatic hydrocarbons and metallic ions which are major components of petroleum products. For this reason, the toxicological effect of the water soluble fraction (WSF) of Bonny light crude oil (BLCO) was evaluated. Methods: The range finding test was determined to be higher than 100% and showed no mortality or physical changes after 7 days. Wistar albino rats were exposed to three different concentrations (25, 50 and 100%) of WSF (BLCO) for a period of 28days. Results: Data from the study showed a significant ($p \leq 0.05$) increase in liver marker enzymes [aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP)] and biochemical parameters (cholesterol, urea, total and direct bilirubin) in rats exposed to WSF (BLCO). Generally, the increase in the level of biochemical parameters was concentration dependent with rats in the group treated with 100% concentration showing the highest activity when compared with control. There was a marginal decrease in the level of packed cell volume (PCV) and haemoglobin (Hb) in rats exposed to WSF (BLCO). White blood cell (WBC) of rats exposed to 25 and 50 % WSF (BLCO) increased marginally whereas a significant ($p \leq 0.05$) increase was observed in the group exposed to 100% of WSF (BLCO). The histological examination of rats exposed to different concentrations (25, 50 and 100%) of WSF (BLCO) were characterized by fatty change, inflammation of the cell whereas rats in the control group showed normal architecture. Conclusion: The findings of this study highlights the deleterious and toxicological effects of exposure to water polluted by dissolved aromatic hydrocarbons probably present in WSF (BLCO).

KEYWORDS: toxicology; amino acid transferases; crude oil; liver damage.

INTRODUCTION

Crude oil is a naturally occurring substance found in certain rock formations on the earth. It is a dark, sticky liquid classified as a hydrocarbon. It is a complex mixture and vary widely in composition. It is highly flammable and can be burnt to create energy. The water environment experiences many dynamic changes induced by various events such as the spillage of toxic chemical that may have significant impact on life (Camougis, 1981). The severity of the effects depends on the organisms' exposure, the concentration of the components and mode of exposure (Overton *et al*., 1994). Water and oil are usually considered to be non-miscible. However crude oil contains a very small soluble fraction (Kavanu, 1964). The water soluble fraction (WSF) constituents are dispersed particulates oil, dissolved hydrocarbons and soluble contaminants such as metallic ions (Kauss and Hutchinson, 1975). The components of crude oil that go into solution make up the water soluble fraction. They are taken up by living cells and are metabolized (Ali and Mai, 2007). This is ecologically important because in event of oil spill into aquatic habitat, this is absorbed by living organisms with serious effects on the ecosystem. The toxicity of crude oil has been reported by Overton *et al*., (1994) to be due to the fraction of the presence of toxic components like xylene, naphthalene, benzene and toluene. The water-soluble fraction (WSF) of crude oil and their derivatives products contains a mixture of polycyclic aromatic hydrocarbons (PAHs), monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes; phenols and heterocyclic compounds, containing nitrogen and sulfur (Saeed and Al-Mutairi, 1999), and also heavy metals. Some petroleum-derived hydrocarbons are toxic to a wide spectrum of marine animals because they preferentially accumulate in lipidic compartments like cellular membrane (Di Toro *et al*., 2001), disturbing the physicochemical and physiological membrane properties (Sikkema *et al*., 1994). Gunlacks and Hayas (1977) reported that the growth rate and biomass turnover of

some aquatic macrophytes have been adversely affected by the water soluble fraction of crude oil. However, the presence of harmful metallic ions in WSF has been reported (Kauss and Hutchinson, 1975; Winter *et al*., 1976; Noyo *et al*., 2007; Noyo *et al*., 2008). Accumulation of these ions such as Na^+ and Ca^{2+} may result in several stress problems capable of destroying plant cell wall and membranes (Hernandez *et al*., 1995). The combination of these ions with Cl^- in solution to form NaCl and $CaCl_2$ may cause leakage of cell contents and eventual death of cell (Hoagland, 1972). One of the major problems of the inhabitants of the Niger Delta region of Nigeria is the contamination of water and aquatic lives by crude oil. This contamination may not necessarily lead to outright mortality but may have significant effects which can lead to physiological stress and dysfunction in animals. The severity or degree of the problems in the inhabitants of the area is dependent upon the point of contact with the polluted water. Hence, the need for the preparation of different crude oil concentrations. Limited information on the impact of exposure of terrestrial animals to dissolved hydrocarbon such as water soluble fraction of crude oil was the motive for the present study. However, this study is an attempt to evaluate the toxicological effects of the water soluble fractions (WSF) of Bonny light crude oil on Wistar albino rats.

MATERIALS AND METHODS

Collection of Samples

Fresh samples of Bonny light crude oil (BLCO) were collected from the N.N.P.C Refinery at Eleme, Rivers State, Nigeria.

Preparation and preservation of the WSF (BLCO).

The water-soluble fraction was prepared according to the method of Anderson *et. al.,* 1974 with slight modification as described by Ogali *et al.,* (2007). Briefly, A sample of bonny light crude oil (BLCO) (150 ml) was slowly mixed with distilled H_2O (450 ml) in a 1000- ml conical flask. The flask was covered with Aluminum foil and held tightly with a rubber band. The flask was fastened to an electric stirrer, and shaken for 24 h as recommended by Parker *et al*., (1976) and adopted by Patrick-Iwuanyanwu *et al*., 2010. Then, the mixture was left standing for 3 h to obtain a clear phase separation between crude oil and H_2O. The mixture was then poured into a separating funnel (with glass stopper) and allowed to settle overnight. The pure and clear WSF obtained at the lower part of the funnel was collected into a dark-colored, screw-capped Winchester bottle as 100% WSF stock. The stock was further diluted with distilled water to give 50 and 25% concentration WSF and stored in a dark-colored, screw-capped Winchester bottle in a refrigerator (0–4 ^oC) until required for use.

Range Finding Tests

Range finding tests to determine the lowest dose of WSF of Bonny light crude oil capable of eliminating 50% of the test animals and the highest concentration that will not have any effect on the animals were first carried out. Five different concentrations (100, 30, 9, 2.7 and 0.81) of the WSF of the crude oil were used based on a dilution factor of 0.3. Animals were closely monitored for 7days for observational changes such as discharges from the eyes, nose, hair loss, tremors, changes in respiratory rate and movement within the cage.

Animals

Thirty two matured Wistar albino rats weighing between 170–180g used in this experiment were obtained from the Animal House of the Department of Biochemistry, University of Port Harcourt, Nigeria. They were housed and kept under laboratory conditions with free access to a standard diet and water for seven (7) days of acclimatization. The experiment was performed after the experimental protocol was approved by the institutional animal ethics committee

Experimental Protocol

After the acclimatization period, the rats were randomly selected into four groups comprising of eight animals each. Rats in group I were fed with normal feed and water only (Control group) whereas rats in group II were fed with normal feed, water and 1ml of 25% of WSF (BLCO) orally daily for 28 days while rats in group III were fed with normal feed, water and 1ml of 50% WSF (BLCO) orally daily for 28 days and Group IV was treated with normal feed, water and 1ml of 100% WSF (BLCO) orally daily for 28 days.

Sample collection

Twenty four hours after the 28 days of oral administration of WSF (BLCO), the rats were anaesthetized in a chloroform-saturated chamber after which the animals were sacrificed using cervical dislocation method. Blood samples were obtained by cardiac puncture from each rat by means of a 2 ml hypodermic syringe and needle. The blood samples were introduced into clean dry bottles (EDTA bottles) for haematological parameters while the blood samples used for biochemical parameters were collected in an anticoagulant free bottle. Serum was separated by centrifugation at 2500rpm for 10 minutes and stored in a refrigerator at 4 ^oC until use. The levels of biochemical parameters (ALT, AST, ALP, total and direct bilirubin, cholesterol and urea) were estimated using the Humazym MUV test kits. The white blood cells (WBC) were estimated using the improved Neubauer counting chambers as described by Dacie and Lewis (1991). The haemoglobin (Hb) concentration was determined by the Cyameth-haemoglobin method while the Packed Cell Volume (PCV) was determined by the micro method as described by Dacie and Lewis (1991).

Table 1: Treatments groups used in this study

Group	Treatment	Duration	No of rats
Group (I)	Normal feed + H_2O	28 days	8
Group (II)	Normal feed + H_2O + 25% WSF	28 days	8
Group (III)	Normal feed + H_2O + 50% WSF	28 days	8
Group (IV)	Normal feed + H_2O + 100% WSF	28 days	8

Histopathological Examination

A portion of the liver of all the rat groups was fixed in 10% buffered neutral formalin for 48 hours followed by bovine solution for 6 hours and then processed for paraffin embedding. By using a microtome, sections of 5 µm

thickness were taken, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin (Galigher and Kayloff, 1971) and subjected to histopathological examination.

Statistical analyses

The results are expressed as mean ±standard error of the mean (SEM). One-way analysis of variance (ANOVA) was employed for between and within group comparison while student's t-test was used for paired comparison. 95% level of significance ($p \leq 0.05$) was used for the statistical analysis.

RESULTS

The results of the range finding test showed no mortality or physical changes such as discharges from the eyes, nose, hair loss, tremors, changes in respiratory rate and movement within the cage after 7 days. The range finding test was however determined to be higher than 100% after 7 days. The results of the effect of oral administration of the Water soluble fraction (WSF) of bonny light crude oil (BLCO) at 25, 50 and 100% on liver enzymes are shown in Table 2. Results from the study showed significant ($p \leq 0.05$) increases in AST, ALT and ALP activities in the treated groups with rats in the group administered 100% showing the highest activity when compared with control (Table 2). Administration of the WSF (BLCO) at 25, 50 and 100% strength to experimental rats significantly ($p \leq 0.05$) increased total and direct bilirubin with rats treated with 100% WSF showing the highest values (28.30±0.33 and 14.40±0.03U/L) when compared with control (13.10±1.76 and 6.50±0.83U/L) for total and direct bilirubin respectively. The effect of the WSF on cholesterol and urea are shown in Table 2. Results of the study showed a significant ($p \leq 0.05$) increase in the level of cholesterol and urea in groups treated with 25, 50 and 100% of the WSF when compared with control. The result of the effect of the WSF (BLCO) on haematological parameters is presented in Table 3. The PCV and Hb level in the treated groups showed marginal decrease when compared with the control. However, there was a marginal increase in WBC of rats treated with 25 and 50% of the WSF (BLCO) whereas rats treated with 100% of the WSF (BLCO) showed a significant ($p \leq 0.05$) increase when compared with control. Results of the histopathological examination of the liver are shown in Figures 1-4. The result of the study on the liver of rats in the control group showed normal architecture of hepatocytes whereas hepatocytes of rats in the groups administered 25, 50 and 100% WSF(BLCO) were characterized by fatty change, inflammation of the cells around the portal tract (Portal Trinitis) and apoptosis of cell (Figures 2-4).

DISCUSSION

One of the major problems of the inhabitants of the Niger Delta region of Nigeria is the contamination of water and aquatic lives by crude oil spills. This contamination may not necessarily lead to outright mortality but may have significant effects which can lead to physiological stress and dysfunction in animals. The toxicity of a petroleum fraction is related to its hydrophobicity (Freedman, 1995) because lipid solubility is an important factor in the passage of petroleum components through the plasma membrane of cells, as well as the degree of membrane disruption. The result in this study clearly indicate that oral administration of different concentrations

Table 2: Effect of oral administration of WSF (BCLO) on serum biochemical parameters of rats

%WSF	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	7.33±0.88	6.33±0.67	15.67±1.20
25%	15.33±0.67[a]	16.00±1.53[a]	24.00±0.58a
50%	19.00±2.00[b]	17.67±1.20[a]	25.67±0.33[b]
100%	25.33±0.67[c]	22.67±0.88[b]	27.33±0.33[b]

%WSF	Total bilirubin (μmol/L)	Direct bilirubin (μmol/L)	Cholesterol (Mmol /L)	Urea (Mmol/L)
Control	13.10±1.76	6.50±0.83	2.40±0.10	5.30±0.14
25%	18.70±0.65[a]	9.60±0.27[a]	2.50±0.11[a]	6.00±0.14[a]
50%	24.60±2.40[b]	12.00±1.07[b]	6.80±0.03[b]	6.80±0.03[b]
100%	28.30±0.33[c]	14.40±0.03[c]	2.90±0.03[b]	8.10±0.85[c]

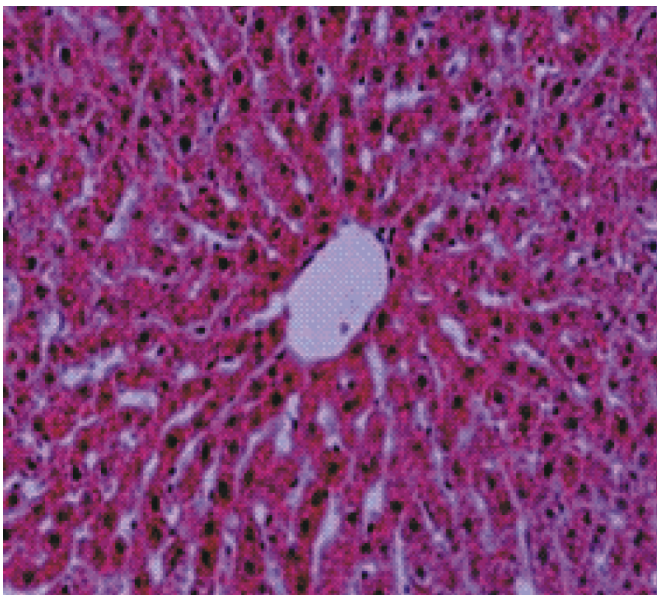

Figure 1: A section of the rat liver tissues showing normal architecture in the control rats.

(25, 50 and 100%) of the water soluble fraction (WSF) of Bonny Light Crude Oil (BLCO) for 28 days resulted to a signifigant ($p \leq 0.05$) increase in the levels of biochemical parameters. The increased levels of aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) are conventional indicators of liver injury (Shah *et al.*, 2011). These serum enzymes (ALT

Table 3: Effect of oral administration of WSF (BCLO) on haematological parameters of rats

%WSF	PCV (%)	HB (g/dl)	WBC (cell mm^{-3})
Control	31.80±1.11	9.14±0.29	5200±70.71
25%	29.75±0.28	8.73±0.09	5250±76.32
50%	28.50±1.03	8.43±0.48	5275±85.93
100%	27.25±1.44	8.20±0.64	5425±95.74

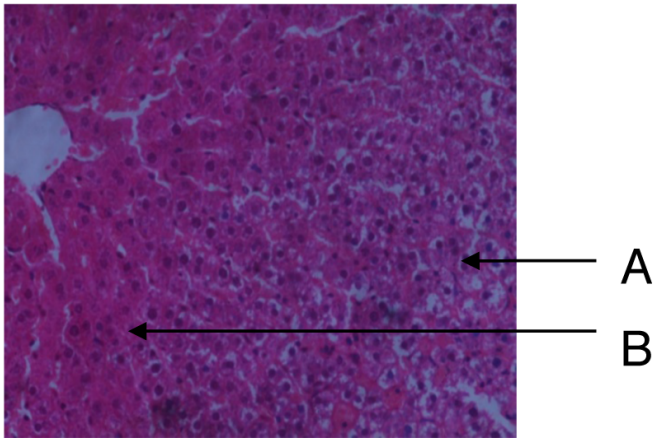

Figure 2: A section of the rat liver administered with 25% WSF (BLCO) showing fatty change (A) and cells round the portal tract spared (B).

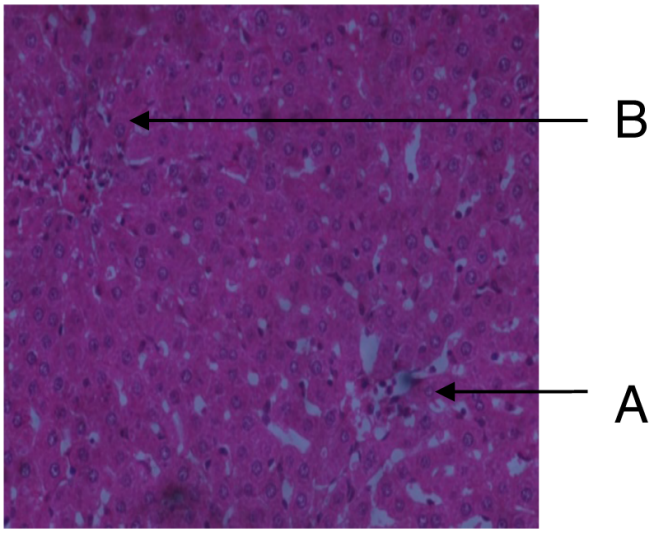

Figure 3: A section of the rat liver administerd with 50% WSF (BLCO) showing fatty change, inflammation of cells round the portal tract (Portal Trinitis) (A) and Apoptosis-Death of cells (B).

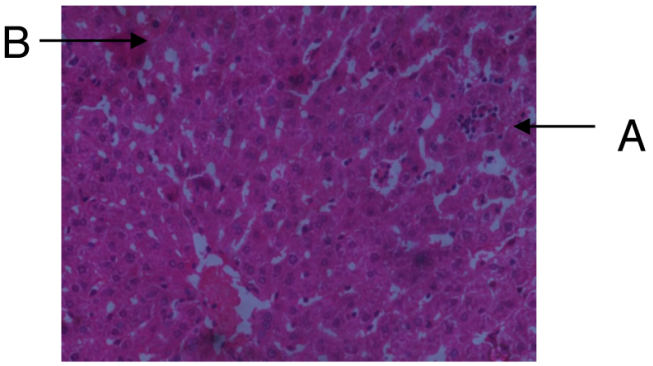

Figure 4: A section of the rat liver administered with 100%WSF (BLCO) showing mild fatty change, mild inflammation (A) of the Cell round the portal tract and Apoptosis of cell (B).

and AST) are largely used in the assessment of liver damage by drugs or any other hepatotoxin (Rahmaiah, 2011; Patrick-Iwuanyanwu *et al*., 2012). The elevation of serum marker enzymes observed in this study may be attributed to severe hepatocellular injury.

The rise in the enzyme AST with a corresponding increase level of ALT observed in this study corroborates the findings of Sallie *et al*., 1999. High AST level is an indicator of liver damage (Crook, 2006). Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in the liver (Drotman and Lawhan, 1978). This cellular leakage may be attributed to harmful metallic ions and dissolved hydrocarbons present in WSF (BLCO) (Kauss and Hutchinson, 1975; Winter *et al*., 1976) which are capable of destroying cellular membranes (Hernandez *et al*., 1995). The elevated conjugated bilirubin level observed in rats treated with different concentrations of WSF (BLCO) may be an indication of hepatobiliary disease. The increase in the level of urea observed in this study probably indicates that the WSF (BLCO) interfered with the renal function capacity to excrete this metabolite. This further indicates that renal integrity of rats treated with WSF (BLCO) may have been adversely affected. The increase in cholesterol levels may probably be an indication of liver damage. Haematological indices such as haemoglobin (Hb), packed cell volume (PCV) and white blood cell (WBC) provide information on the general state of the blood of an organism at a particular time. They are often associated with health indices and are of diagnostic significance in routine clinical evaluation of the state of health (Patrick-Iwuanyanwu *et al*., 2007). The result from the present study showed a dose dependent decrease in Hb and PCV in rats treated with WSF (BLCO). This finding is similar to the report by Ovuru and Ekweozor (2004) in rabbits, Leighton *et al*., (1985) in young Herring gulls and Atlantic Puffins. The result from this study has demonstrated that long-term exposure to WSF (BLCO) samples induces anaemia. The resulting anaemia is in accordance with the report of Krishna and Veena (1980) who reported the suppressive effect of petroleum samples on erythropoiesis. Reports of Sudakov (1992) and Marieb (1995) have shown that the toxic components especially those in

petroleum products change blood chemistry and hence induce anaemia by causing bone marrow hypoplasia and interfered with platelets production in the animals, hence the reduced values of Hb and PCV in rats treated with WSF (BLCO). The decrease in Hb and PCV levels in the treated rats is an indication that the WSF (BLCO) was capable of eliciting haemolytic toxicity of the blood cells in condition of long-term exposure. This may be attributed to cytotoxic effect and suppression of erythropoiesis caused by constituents of the WSF (BLCO). Crude oil fraction present in the WSF may be responsible for the serious consequences on hematological parameters in the experimental rats. The white blood cell (WBC) functions primarily in body defense against foreign bodies. This is achieved by leucocytosis and antibody production (Robins and Angell, 1976; Marieb, 1995). However, the increase in the level of WBC may be attributed to the defensive mechanism of the immune system (Hoeney, 1985).

Histopathological examinations of the liver tissues of the experimental rats indicate that exposure to WSF (BLCO) affected the structural integrity of the liver cells.This is characterized by the presence of fatty change, inflammation of cells round the portal tract (portal trinitis) and Apoptosis (death of cells).This implies that the liver is one of the major target organs of WSF (BLCO) -induced injury.The cumulative oxidative damage is therefore likely to be one of the underlying mechanisms responsible for the hepatotoxic effects of WSF (BLCO) as observed in the study.

Conclusion

In conclusion, the results of this work suggest that repeated exposure to WSF (BLCO) may elicite an increase in serum enzyme activities and biochemical parameters (cholesterol, urea, total and direct bilirubin). This may be attributed to the toxicity of dissolved hydrocarbons and metallic ions present in WSF (BLCO). It then implies that long term exposure to WSF (BLCO) may be hepatotoxic, nephrotoxic and haematotoxic.

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