

Full Length Research Paper

Haematological and immunological effect of co-administration of extracts of *Vernonia amygdalina* and *Azadirachta indica* on normal and diabetic rats

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This study evaluated the effect of co-administration of extracts of *Vernonia amygdalina* Del. (VA) and *Azadirachta indica* Linn.(AI) on haemopoietic and immunological indices of normal and diabetic rats. White blood cells which were non-significantly decreased ($p>0.05$) in diabetic control rats relative to the normal control, respectively increased and decreased non-significantly ($p>0.05$) upon administration of the combined extracts of VA and AI to diabetic and non-diabetic test rats. Packed cell volume, haemoglobin content and red cell count as well as its derived factors (mean cell volume, mean cell haemoglobin and mean cell haemoglobin concentration) of both diabetic and non-diabetic rats were not affected by the treatment, relative to their respective controls. As compared to insulin treatment, the combined extracts significantly increased ($p<0.05$) mean cell haemoglobin concentration (MCHC) of diabetic rats. Platelets number which was non-significantly lower ($p>0.05$) in diabetic control rats as compared to non-diabetic control was further decreased non-significantly ($p>0.05$) upon administration of the combined extracts and insulin. Diabetes induction significantly increased CD_4^+ count ($p<0.05$) as compared to the normal control. This was however decreased significantly ($p<0.05$) upon treatment with the combined extracts and insulin. The combined extract similarly decreased CD_4^+ counts in normal test rats as compared to the normal control. Combined extracts of VA and AI is non-haematotoxic and may possess some anti-inflammatory properties when used as a management against diabetes mellitus.

Key words: Diabetes, haematological and immunological indices, *Vernonia amygdalina*, *Azadirachta indica*.

INTRODUCTION

Since ancient times, plants have been exemplary source of medicine (Grover et al., 2002) and have played key roles in traditional health care systems and also form the basis of a significant percentage of allopathic and modern drugs in industrialised nations of the world (Calson, 1998; Samy and Gopalakrishnakone, 2007). In Africa alone, up to 80% of the population uses traditional medicine for primary healthcare, whereas in industrialised parts of the world its counterpart or adaptations also exist in various forms called Complementary and Alternative Medicine (CAM) (WHO, 2003). The World Health Organisation consequently launched its first ever comprehensive

traditional medicine strategy in 2002 to assist countries develop national policies on evaluation and regulation; create a stronger evidence base on safety, efficacy and quality; ensure availability and affordability; promote therapeutically sound use; and document them especially in diseases including HIV/AIDS, malaria, sickle cell anaemia and diabetes mellitus (WHO, 2003).

In line with the aforementioned, numerous herbs have not just been screened for their hypoglycaemic and antihyperglycaemic activities, but have gained approval of several cooperate bodies and organisations to be further researched into. The believe is that medicinal preparations either from a single herb or a combination of herbs are holistic in therapeutic approach and may be best suited for a syndrome such as diabetes with multiple pathologies (Tiwari and Rao, 2002). More so, they could

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Table 1. Experimental design.

Group	Number of animal	Treatment
Non-diabetic batch		
NC	6	Placebo (non diabetic control)
NVA	6	<i>V. amygdalina</i> extract (200 mg/kg b.w)
NAI	6	<i>A. indica</i> extract (200 mg/kg b.w)
NVA/AI	6	<i>V. amygdalina</i> and <i>A. indica</i> combined extracts (100 mg/kg b.w each)
NHU	6	Insulin (5 unit/kg b.w)
Diabetic batch		
DC	6	Placebo (diabetic control)
DVA	6	<i>V. amygdalina</i> extract (200 mg/kg b.w)
DAI	6	<i>A. indica</i> extract (200 mg/kg b.w)
DVA/AI	6	<i>V. amygdalina</i> and <i>A. indica</i> combined extracts (100 mg/kg b.w each)
DHU	6	Insulin (5 unit/kg b.w)

be better dietary adjuncts in the establishment and maintenance of euglycemia since plant foods are thought to have acquired biological adaptations to the human system, hence have minimal side effects (Srinivasan, 2005).

The comparative antidiabetic activity of two plants used in traditional management of diabetes: *Vernonia amygdalina* Del. and *Azadirachta indica* A. Juss, was recently investigated in our laboratory (Ebong et al., 2006). Later, enhanced efficacy in glycaemic control and reduced toxicity effect of combined extracts from the two plants and individual extracts in alloxan diabetic rat models was reported (Ebong et al., 2008). In furtherance of these previous reports, we in this investigation evaluated the effect of combined extracts of these plants (*V. amygdalina* and *A. Indica*) on haematological and immunological indices of normal and streptozotocin diabetic rats, with a view to ascertain the effect of the potential anti-diabetic option on complications of haemopoietic and immune systems usually engendered by chronic diabetes mellitus.

MATERIALS AND METHODS

Collection of plant materials

Matured leaves of *V. amygdalina* (Del.) and *A. indica* (A. Juss) were respectively collected from the Endocrine Research Farm and the Staff Village, University of Calabar. These were duly authenticated and voucher specimens were deposited in a herbarium (ERU/2006/011 and ERU/2006/012, respectively) in the Department of Botany. The leaves were rinsed severally with clean tap water to remove dust particles and debris and thereafter allowed to completely drain.

Preparation of plant extracts

The plant materials were separately chopped with a knife on a chopping board and (1 kg) each of *A. indica* and *V. amygdalina* was

homogenized with an electric blender in 1.95 and 2.25 L of 80% (v/v) ethanol, respectively. The mixtures were then allowed for 48 h in a refrigerator at 4°C for thorough extraction of the plants' active components. These were then filtered with cheesecloth and later with Whatman No. 1 filter paper to obtain a homogenous filtrate. The filtrates were then concentrated *in vacuo* at low temperature (37 to 40°C) to about one tenth of the original volume using a rotary evaporator. The concentrates were further allowed open in a water bath (40°C) for complete dryness yielding 40.54 g (4.054%) and 34.71 g (3.471%) of greenish brown and brown oily substances for *V. amygdalina* and *A. indica*, respectively. The extracts were then refrigerated at 2 to 8°C until use.

Animals and experimental design

60 male albino rats of Wistar strain weighing about 140 to 180 g were obtained from the animal house of the Department of Zoology and Environmental Biology, University of Calabar, Calabar. The animals were allowed to acclimatize for three weeks in the animal house of the Department of Biochemistry during which they were housed in well ventilated cages (wooden bottom and wire mesh top) and kept under controlled environmental conditions of temperature (25 ± 5°C), relative humidity (50 ± 5%) and 12 h light/dark cycle. The 60 rats were divided into five parallel groups consisting of a diabetic and a non-diabetic batch, each with six animals per group (Table 1).

Induction of experimental diabetes

Prior to diabetes induction, the rats were subjected to 12 h fast, and then diabetes was induced by intraperitoneal injection of 65 mg/kg b.w. with streptozotocin (STZ) (Sigma St. Louis, MO, U.S.A). Control animals received saline only. Seven days after STZ treatment, diabetes was confirmed in STZ treated rats with a fasting blood sugar concentration ≥200 mg/dl. This was estimated using One Touch® Glucometer (Lifescan, Inc. 1995 Milpas, California, U.S.A) with blood obtained from the tail vein of the rats.

Experimental protocol

Diabetic and non-diabetic animals were grouped as shown in Table 1 and accordingly, treated with extracts and insulin. The dosages of

Table 2. Effect of treatment on haematological and immunological indices of non diabetic rats.

Group/ Treatment	WBC (10 ³ /μl)	RBC (10 ⁶ /μl)	HB (g/dl)	PCV (%)	MCV (f/l)	MCH (pg)	MCHC (g/dl)	PLT (10 ³ / μl)	MPV (f/l)	LYM (10 ³ / μl)	CD ₄ (cells/ μl)
NC	19.52 ±5.45	7.54 ±0.26	11.37 ±0.41	46.62 ±2.36	61.65 ±1.18	15.07 ±0.10	24.50 ±0.50	882.70 ±84.33	7.74 ±0.23	10.70 ±1.79	14.83 ±4.19
VA _{ND}	14.46 ±0.95	7.54 ±0.24	11.54 ±0.47	48.82 ±1.43	64.74 ±0.62	15.28 ±0.22	23.60 ±0.30	1033.20 ±76.42	7.72 ±0.14	10.12 ±0.84	6.80 ±0.49 ^b
AI _{ND}	13.60 ±2.92	7.19 ±0.19	11.17 ±0.28	47.02 ±1.39	65.50 ±1.74 ^b	15.55 ±0.26	23.77 ±0.27	691.17 ±60.49 ^{c,d}	8.25 ±0.19	10.95 ±2.73	7.50 ±1.18 ^b
VA/AI _{ND}	17.35 ±3.05	7.48 ±0.31	11.20 ±0.35	46.98 ±1.46	62.90 ±1.16	15.00 ±0.31	23.88 ±0.35	937.40 ±56.27	7.78 ±0.07	10.90 ±2.11	8.20 ±1.39 ^b
HU _{ND}	13.74 ±2.74	7.43 ±0.38	11.30 ±0.18	47.54 ±0.87	63.86 ±0.62	15.20 ±0.23	23.78 ±0.23	1022.00 ±126.11	8.14 ±0.27	10.40 ±2.78	9.80 ±0.58

Mean ± SE, n = 6; ND = non diabetic; D = diabetic; HU = insulin; a = p < 0.05 vs DC; b = p < 0.05 vs NC ; c = p < 0.05 vs VA/AI ; d = p < .05 vs HU.

the plant extracts were as determined from preliminary work in our laboratory, whereas insulin dose, NPH (5 U/kg b.w. s.c.) was as previously used by Sonia and Srinivasan (1999). The plant extracts were administered via gastric intubation, twice per day (6.00 am and 6.00 pm) and insulin once per day post prandial (6.00 pm) for 28 days. The animals were maintained on pelletised growers mash (obtained from Vital Feeds, Jos, Plateau State, Nigeria) and tap water. Both the feed and water were provided *ad libitum* and environmental conditions maintained as stated earlier throughout the 28 days.

Collection of samples for analysis

At the end of the 28 days, food was withdrawn from the rats and they were fasted overnight but had free access to water. They were then euthanized under chloroform vapour and sacrificed. Whole blood was collected via cardiac puncture using sterile syringes and needles. The whole blood was emptied into EDTA containers preparatory to CD₄⁺ and haematological (full blood count) analysis.

Full blood count assays

Full blood counts including PCV, Hb, RBC, WBC, platelet count, differential WBC (lymphocytes and mixed), and red cell indices (MCHC, MCH and MCV), were estimated using the Sysmex[®] Automated Haematology Analyzer KX-21N, Sysmex Corporation, Kobe-Japan. The pre-diluted (PD) sample method was used where blood was diluted manually, and then fed into the transducers. The transducer chamber has a minute hole called the aperture. On both sides of the aperture, there are electrodes between which flows direct current. Blood cells suspended in the diluted sample pass through the aperture, causing direct current resistance to change between the electrodes. As direct current changes, the blood cell size is detected as electric pulses. Blood cell count is then calculated by counting the pulses, and a histogram of blood cell sizes is plotted by determining the pulse sizes. Also, analyzing a histogram makes it possible to obtain various analysis data including differential whole blood count, red cell indices and derived values.

CD₄⁺ count

The CD₄⁺ lymphocytes were estimated using the Cyflow Automated

Cell Counter (Partec, Germany). 10 μl of CD₄⁺ PE antibody (Partec, Germany) was mixed with 50 ml of EDTA anticoagulated whole blood in a test tube. The mixture was incubated in the dark chamber for 15 min at room temperature of 22 to 28°C. During incubation, the content of the tube was mixed every 5 min. 800 μl of buffer solution was added, mixed and plugged into the counter. The CD₄⁺ cells were separated from monocytes and noise and result were recorded.

Statistical analysis

The results were analysed for statistical significance by one way ANOVA using the SPSS statistical program and Post Hoc Test (LSD) between groups, using MS excel program. All data were expressed as mean ± SEM. P values <0.05 are considered significant.

RESULT

Presented in Tables 2 and 3 are the haematological and immunological indices of non diabetic and diabetic rats, respectively, that received various treatments for 28 days. White blood cells (WBC) which were non-significantly decreased (p>0.05) in diabetic control rats relative to the normal control, became increased non-significantly (p>0.05) upon administration of the extracts of VA only, combination of VA and AI, and insulin as compared to the diabetic control. Similar treatments in the non-diabetic rats rather produced a non significant reduction (p>0.05) in WBC relative to the non-diabetic control. Red blood counts (RBC), haemoglobin (HB) content, packed cell volume (PCV) and their derived factors-mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) of both diabetic and non-diabetic test groups were not affected by the different treatments, except for AI extract which significantly increased (p<0.05) HB in diabetic test rats and MCV in non diabetic test rats when respectively compared to insulin treated and non diabetic control groups. Also, MCHC levels of diabetic test rats treated with extracts of

Table 3. Effect of treatment on haematological and immunological indices of diabetic rats.

Group/ Treatment	WBC (10 ³ /μl)	RBC (10 ⁶ /μl)	HB (g/dl)	PCV (%)	MCV (f/l)	MCH (pg)	MCHC (g/dl)	PLT (10 ³ / μl)	MPV (f/l)	LYM (10/ μl)	CD ₄ (cells/ μl)
DC	14.00 ±1.82	7.19 ±0.37	10.85 ±0.63	44.4 ±2.57	61.75 ±1.50	15.05 ±0.27	24.40 ±0.23	688.60 ±83.54	8.08 ±0.29	11.68 ±1.76	51.50 ±10.17
NC	19.52 ±5.45	7.54 ±0.26	11.37 ±0.41	46.62 ±2.36	61.65 ±1.18	15.07 ±0.10	24.50 ±0.50	882.70 ±84.33	7.74 ±0.23	10.70 ±1.79	14.83 ±4.19 ^a
VA _D	17.50 ±3.08	7.76 ±0.53	11.82 ±0.81	47.32 ±3.20	60.98 ±0.56	15.16 ±0.31	24.98 ±0.34 ^d	864.40 ±106.35	7.90 ±0.18	11.93 ±0.32 ^d	28.20 ±1.53 ^{a,d}
AI _D	12.15 ±2.80	7.90 ±0.35	13.18 ±1.88 ^d	48.60 ±2.70	61.48 ±1.12	15.08 ±0.32	24.55 ±0.26	750.00 ±115.58	7.93 ±0.25	9.68 ±2.84	20.25 ±1.80 ^a
VA/AI _D	16.00 ±2.24	7.48 ±0.11	11.55 ±0.24	46.80 ±1.01	61.53 ±0.62	15.43 ±0.10	25.10 ±0.08 ^d	657.25 ±125.67	7.83 ±0.19	12.00 ±1.27 ^d	20.50 ±3.52 ^a
HU _D	15.86 ±3.63	7.18 ±0.47	10.60 ±0.73	44.70 ±2.68	62.46 ±1.18	14.78 ±0.30	23.66 ±0.42	683.20 ±53.66	8.20 ±0.33	7.68 ±0.82	16.20 ±1.24 ^a

Mean ± SE, n = 6; NC = normal control; DC = diabetic control; HU = insulin; a = p<0.05 vs DC; b = p<0.05 vs NC; c = p<0.05 vs VA/AI; d = p<0.05 vs HU.

VA alone, and VA and AI combined together were significantly higher ($p < 0.05$) than insulin administered group. Platelet counts in diabetic rat were non-significantly lower than the normal control rats. Whereas administration of VA and AI extracts singly caused increased platelet number, combined extracts and insulin treatments showed further decreases. However, these changes were non significant ($p > 0.05$). Besides, AI extract treatment which caused significant decrease ($p < 0.05$) in platelets as compared to the combined extracts and insulin in non-diabetic rats, other test groups showed non significant changes. There was significant increase in CD₄⁺ counts ($p < 0.05$) of diabetic rats relative to non-diabetic rats. These were significantly decreased in all diabetic test groups ($p < 0.05$). In non-diabetic test groups also, CD₄⁺ levels was significantly decrease ($p < 0.05$) following treatments with the extracts of VA, AI alone, and VA and AI combined together, relative to the non diabetic control. The decrease in insulin treated group was however not significant.

DISCUSSION

Anaemia in diabetes, atherosclerosis resulting from increased platelet aggregation, glycosylation of haemoglobin (RBC) and of recent, white blood cells, are some terms discussed extensively in diabetes (Colwell and Nesto, 2003; Thomas et al., 2005; Nakagami et al., 2005; Yamagishi et al., 2005). We therefore assayed in our study, haemopoietic status and some immune system indices.

WBC, RBC and PCV were seen to be decreased in the untreated diabetic rats as compared to the non-diabetic. These could result from onset of glycosylation process in the untreated diabetic rats or ensuing anaemia of diabetes. Anaemia has been identified as a common

complication of chronic kidney disease (CKD), affecting over half of all patients (USRDS, 2002) and the most common cause of CKD in about $\frac{2}{3}$ of cases is diabetes mellitus (Thomas et al., 2005). These authors in an earlier study indicated that approximately 7% of patients with diabetes had haemoglobin level less than 11 g/dl (Thomas et al., 1998). Though the diabetic subjects in this investigation were rats, our report of 10.85 ± 0.63 g/dl haemoglobin level is in accordance with these authors' findings. This was reflected also in PCV and RBC as would be expected. The non significance in these differences could be attributed to the duration of the study (28 days). This period may have been too short for pronounced pathogenesis required for full expression of anaemia— glycosylation or advanced glycosylation end products AGEs formation and end stage damage of kidney to impair erythropoietin synthesis or formation. Our observation as per VA extracts contrast Oboh (2001), who had reported decreased RBC and PCV, due to induced haemolysis in normal rats treated with saponin extracts from VA leaves. When used as whole extracts, other constituents in the leaves of VA or AI used in combination, may have interacted synergistically to suppress the haemolytic effects of the saponins. The observed slight reduction in WBC in AI extract treated rats both in diabetic and non-diabetic rats does not imply reduced immunity since the result of the lymphocyte count compared well with that of the non-diabetic control rats. According to Leob (1991), WBC count may vary by as much as 2,000 due to strenuous exercise, stress or digestion and is diagnostically useful only when interpreted in light of the white cell differential count.

Platelet counts appeared to have decreased slightly in the diabetic control relative to the non-diabetic. Thrombocytopenia is usually seen as destruction caused by drugs or immune disorders. There appear to be an immune disorder from our study, as seen clearly in the CD₄⁺

counts and the decrease in platelet counts. Also, decrease in circulating platelet number may also mean an increase in aggregated platelet typical of atherosclerotic disorders including diabetes mellitus. Increased platelet activation and subsequent aggregation favouring thrombosis has been extensively discussed (Colwell and Nesto, 2003; Brand et al., 1998). Prostacyclin and nitric oxide which inhibit this process in normal endothelium are reportedly reduced in people with diabetes (Klein and Colwell, 1996; Martina et al., 1998). The observed increase with VA extract treatment in diabetic and non-diabetic rats may be connected with decrease in aggregability engendered by the antioxidant property of the plant. Platelets from diabetics have been reported to contain reduced antioxidant levels (Sarji et al, 1979). It is not clear however why the observation was not similar with AI extract and combined extracts of VA and AI.

The elevated CD₄⁺ count in diabetic controls as compared to non-diabetic control observed in this study is a probable inflammatory response to atherosclerotic pathogenesis of diabetes. Blood-borne immune cells constitute an important part of atheroma, the core of which is infiltrated by T-cells and predominantly the CD₄⁺ T cells (Hansson, 2005). Metabolic breakdown in diabetes usually results in increased circulation and deposition of lipids in arteries; atherosclerosis (Hansson, 2005), hence an increase in immune and inflammatory response which eventually leads to elevation in associated T-cell variant, CD₄⁺. Reduction in CD₄⁺ counts by administration of extracts of AI and VA singly and in combination would not be unconnected with their anti-inflammatory effects. The anti-inflammatory properties of AI (Biswas et al., 2002) and VA (Farombi, 2003) have extensively been reviewed. These effects were also demonstrated in non-diabetic rats as opposed to insulin. In conclusion, administration of the extracts of VA and AI singly and in combination may therefore be more potent as anti-inflammatory agents than insulin in the management of diabetes mellitus.

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