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Original Article

ENHANCEMENT OF INSULIN AND REPRODUCTIVE HORMONE LEVELS BY COMBINED LEAF EXTRACTS OF VERNONIA AMYGDALINA AND GONGRONEMA LATIFOLIUM IN DIABETIC MODELS

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Abstract: The study evaluated the effects of combined extracts of Vernonia amygdalina (VA) and Gongronema latifolium (GL) on the pancreas of streptozotocin (STZ) induced diabetic Wistar rats and on serum concentrations of insulin and reproductive hormonal levels. Thirty-six (36) albino rats were divided equally into 6 groups. Groups A and B which served as normal (NC) and diabetic (DC) controls respectively were only administered placebo. The diabetic test groups C, D, E and F were respectively treated with metformin (5mg/kg b. w.), combined extracts of VA and GL (400mg/kg b.
w.), VA (200mg/kg b. w.) and GL (200mg/kg b. w.), for 28 days. Thereafter, the animals were sacrificed; blood and pancreas were collected for serum hormonal assays and histological evaluation, respectively. Changes in animal weights and blood glucose concentrations were also measured within the experimental period. From the results it was observed that the body weight of the animals in both the VA and metformin treatment groups was significantly increased ($p<0.05$) from -10.5% reduction in the DC, to 10.7% and 23.3% respectively. In the same order, serum glucose levels significantly decreased ($p<0.05$) by 52.71% and 48.93% in the metformin and combined extract groups respectively. The decrease in other groups was not as remarkable as that of combined extracts group compared to DC which further increased by 64.57%. The extent of reversal of hyperglycemia in the extract treated animals compared well with the metformin treated group. The biochemical results corroborated with results of histological evaluations: The pancreatic β-cells of DC animals which were distorted and degenerated with shrunken cell mass as against prominent islet cells with normal exocrine pancreas of NC animals became rapidly proliferated upon intervention with the combined extracts, suggesting a possible regeneration of the islet cells hence enhanced insulin release which is positively correlated to the observed hypoglycemia and body weight increments . The serum concentration of reproductive hormones of group B animals which was also significantly reduced ($p<0.05$) compared to group A was markedly improved in all the treatment groups. On the other hand, intervention with metformin did not produce observable differences in the cyto-architecture of the pancreatic islets compared to the diabetic control. For the Feulgen's reaction, most of the cells in the groups that received the plant extracts were strongly positive suggesting a reversal of DNA damage in these groups.
Keywords: Vernonnia amygadlina, Gongronema latifolia

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INTRODUCTION

Diabetes Mellitus is a metabolic disorder having the characteristic feature of hyperglycaemia, which is usually due to an absence of insulin, impaired effectiveness of insulin action or tissue insensitivity to insulin (American Diabetes Association, 2014). The elevated blood sugar level is not a pathogenic consequence but a result of the deterioration in endocrine controls (and hence functions). Consequently, diabetes mellitus expresses different features in different stages of its natural history which are usually pathological scenarios requiring different agents if these features are to be managed. Currently, most chemotherapeutic agents in use act only on a segment of the pathology and even still to a biased limit (Luna and Feinglos, 2001), therefore, these agents cannot address the problem in a holistic manner.

Insulin, a hormone produced by the pancreatic beta cells functions to strictly regulate blood glucose concentrations (and is the major hormone implicated in the diabetic state either for its relative absence or for tissues insensitivity to it). The hormone makes it possible for the tissues and cells of the body to make use of glucose for energy provision and in its absence or where its action is impaired, cells and tissues are unable to uptake glucose causing an accumulation of glucose in the blood, and hence the expression of other debilitating complications with associated symptoms as weight loss, excessive thirst, excessive urination, increased hunger and general fatigue are common in diabetic state (Fox, 2004; Larsen *et al.*, 2003).

Diabetes mellitus has also been reported to be one of the causes of sub-fertility/infertility especially among men. In a study by Jiang (1996), it was discovered that approximately 90% of men with diabetes have some form of dysfunction in their sexual life such as erectile dysfunction, reduced libido and infertility. Penson and Wessells (2004) posit that over 71% of diabetic men suffer from erectile dysfunction. Some diabetic men are also said to have normal semen parameters but high levels of mitochondrial and nuclear DNA injury in the sperm cells thus increasing their vulnerability to infertility (Agbaje *et al*., 2007). Also experienced in diabetic men is reduced testosterone levels which may not be unconnected with Leydig cell dysfunction associated with diabetes mellitus (Rehman *et al* 2001). However, the commonest sexual problem in diabetic men is malfunction of erectile tissue.

It has been reported that the use of plant derived extracts may actually treat diabetes mellitus, as these plants contain various herbal and non-herbal properties categorized into phytonutrients and phytochemicals. Some works have even shown that the use of a combination of various agents from different plant sources for therapeutic purposes produces maximum therapeutic efficacy with minimum side effects (Ebong et al., 2008). This study was thus aimed at assessing the capacities of extracts from two indigenous plants (*Gongronema latifolia* and *Vernonia amygdalina*) with wellestablished anti-hyperglyceamic properties at enhancing insulin output in diabetic condition (induced using Streptozocin) and their effects on reproductive hormonal levels.

MATERIALS & METHODS

Plant Collection and Extract Preparation.

Fresh and matured *Gongronema latifolia and Vernonia amygdalina* leaves were bought from Marian market (IkaIkaOqua market) in Calabar municipality. The leaves were washed thoroughly, allowed to completely drain and air dried under shade at ambient temperature. The air dried leaves were homogenized using an electric blender into powder form and thereafter extracted by cold maceration (in methanol solution with intermittent agitations). The filtrate was placed in beakers and allowed to concentrate in a water bath by evaporation at 40°C to total dryness producing about 93g of crude extract each.

Experimental Animals and Induction of Diabetes.

Thirty-six (36) adult albino rats, weighing 80-140g, bought from the Pharmocology Department, University of Calabar. The rats were allowed to acclimatize for two weeks prior to experimentation in the animal house. The animals were kept in properly ventilated cages and at a room temperature of about 27^{o}C with an approximate 12 hour light/dark cycle. Diabetes was induced in overnight fasted experimental animals by a stat dose of

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intraperitoneal injection of newly prepared streptozocin (STZ) 45mg/kg body weight reconstituted in 0.1M sodium citrate buffer (pH4.5-5.0) as solvent. Diabetes was confirmed in the STZ treated rats by checking their fasting blood sugar concentration 48hrs after STZ injection using a glucometer (on-call-plus) and rats having fasting blood sugar above 180mg/dl were regarded to be diabetic and were considered for the research.

STUDY DESIGN

Thirty – six (36) male Wistar rats were used for the study. They were distributed into six groups and treatments administered were as shown in the table below.

Table 1

Experimental design

Groups	Agent administered	Quantity
Normal control (Non diabetic)	Normal tap water	Ad. Libitum
Diabetic control	STZ	45mg/kg
C	Metformin	5mg/kg
D	VA + GL	400mg/kg (200mg/kg Each extract)
E	VA	200mg/kg
F	GL	200mg/kg

TERMINATION OF EXPERIMENT

The experiment was terminated after twenty eight days of administration, after which the animals were sacrificed using chloroform inhalation and the blood and organs collected for biochemical and histological assessments. Blood was collected through cardiac puncture.

HORMONAL ASSAYS

Serum hormonal concentrations for insulin, testosterone, luteinizing hormone and follicle stimulating hormones were estimated by ELISA method using kits from MONOBIND diagnostics according to manufacturers' instructions.

STATISTICAL ANALYSIS

One way analysis of variance (ANOVA) and Post-Hoc Fischer's Least Significant Difference (LSD) tests were used for the statistical analysis. Results are represented as Mean ± Standard Error of Mean (SEM).

RESULTS

Morphological observations: Body weights for the normal control group showed significant ($p<0.05$) weight increase as was also observed in the treatment groups.The combined extract treatment group showed higher percentage of weight increase at the termination of the experiment when compared to basal weights amongst the treatments. (Metformin – 16%, VA+GL – 34%, VA – 13% and GL – 21%). Weight values for the untreated diabetic group (DC) were significantly reduced

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(P<0.05) compared to body weight of the normal control. Also, body weight of the treatment groups (metformin, VA, GL, VA+GL) was significantly increased (p<0.05) compared to the diabetic control (DC).

4.2 Biochemical observations

4.2.1 Blood glucose

Blood glucose concentrations showed significant increase (p<0.05) in the DC group compared to the NC group. The blood glucose levels of all the treatment groups (metformin, combined extracts of VA&GL, VA and GL) was significantly decreased (p<0.05) following treatment compared to the DC group

TABLE 2

Basal body weight and the final body weight of the animals in the various groups

GROUPS	INITIAL BODY WEIGHT (g)	FINAL BODY WEIGHT (g)	% Change in body weight
NORMAL CONTROL	99.91 ± 2.48	136.38 ± 7.28*	36.50
DIABETIC CONTROL	105.73 ± 4.86	94.67 ± 4.43[a]	-10.46
METFORMIN	111.49 ± 9.16	137.62 ± 12.06[b]	23.28
VA + GL	99.77 ± 3.57	123.17 ± 10.11*	27.20
VA	129.00 ± 7.82	142.78 ± 12.30[b]	10.68
GL	106.00 ± 7.88	127.86 ± 10.68	20.62

Data are represented as Mean ± SEM, n=6,

* Significantly different from initial value at p<0.05,

a = Significantly different from NC at p<0.05,b= Significantly different from DC p<0.05,c= Significantly different from initial value p<0.05

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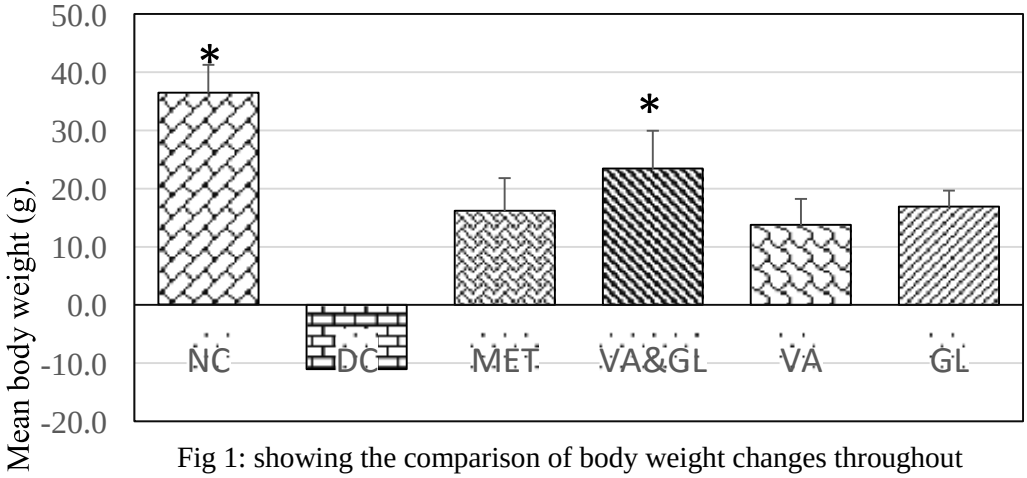

Fig 1: showing the comparison of body weight changes throughout the experimental period.

Data are expressed as Mean ± SEM, n =6

* significantly increased compared to other groups ($p<0.05$)

Key: NC- Normal Control, DC- Diabetic Control, Met- Metformin, VA- *Vernonia amygdalina,* GL- *Gongronema latifolium*

TABLE 3

Mean Basal and Final blood glucose of the disparate experimental arrays

Groups	Initial blood glucose (mg/dl)	Final blood glucose (mg/dl)	% Change in blood glucose
Normal Control	68.16 ± 2.48	60.33 ± 7.28	-11.49
Diabetic Control	156.16 ± 4.86	257.00 ± 4.43[a]	64.57
Metformin	206.40 ± 9.16	97.60 ± 12.06[*]	-52.71
VA+ GL	228.33 ± 3.57	116.60 ± 10.11[*]	-48.93
VA	237.20 ± 7.82	134.20 ± 12.30[*,b]	-43.42
GL	177.16 ± 7.88	105.00 ± 10.68[*]	-40.73

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Data are represented as Mean ± SEM, n=6; * Significantly different from DC at p<0.05; a= Significantly different from NC at p<0.05;b= Significantly different from NC at p<0.05.

Hormonal assays

Table 4 represents data obtained for the mean serum hormone concentrations at the end of the experiment. The level of the various hormones was meaningfully reduced (p<0.05) in the diabetic control group compared to all the other groups. The testosterone level was significantly reduced in the DC group (2.50±0.27) and in the group given the extract of VA alone, the level of the hormone remained significantly low (p<0.05) (3.69 ± 0.37) compared to the normal control (5.87 ±0.28).There was a significant increase in testosterone level in the group treated with Metformin, *Gongronema latifolium* and the combined extracts of VA and GL. For follicle stimulating hormone (FSH), the serum concentration of the hormone was significantly reduced (p<0.05) in all the groups compared to the Normal control (21.77 ± 0.49). However, the level of the hormone was observed to have increased significantly in all the treated groups at the termination of the experiment, compared to the DC group (15.48 ± 0.64). The serum concentration of luteinizing hormone (LH), was significantly reduced in the DC group (19.70 ± 1.18) compared to the NC group (31.62 ± 1.06). At the end of the experiment, all the treated groups recorded meaningful increase in the hormone level compared to the DC. The level of the hormone in the group that received only the GL extract remained significantly low (27.79 ± 1.17) compared to the Normal control. Insulin recorded a meaningful decrease in the DC group (15.07 ± 1.24) compared to normal control (30.69 ± 1.36). In all the treatment groups, the level was significantly increased (p<0.05) analogous to the DC. However, for the groups that received the individual extracts of VA and GL, the level of insulin remained significantly low (23.44 ± 0.18 and 23.99 ± 0.13respectively) compared to the group that received the combined extracts (26.96 ± 1.41). Also it was observed that the level of Insulin in the group that was given with the combined extract of VA and GL was higher compared to all the other treatment groups.

Table 4

Showing the serum levels of the various hormones in the different experimental groups at the end of the experiment.

Groups	**INS (µlu/ml).**	**Testo stero ne (ng/mL).**	**FSH (mlU/ mL).**	**LH (mlU/ mL).**
NC	30.69± 1.36	5.87± 0.28	21.77 ±0.49	31.62 ±1.06
DC	15.07± 1.24*	2.50± 0.27*	15.48 ±0.64	19.70 ±1.18
ME	26.96± 1.41*,a	5.52± 0.77a	19.22 ±0.04	29.23 ±1.12a
TVA	24.29± 0.09*,a	4.37± 0.69	19.54 ±0.03	28.72 ±0.78a

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&	*,a					
G						
L						
V	23.44±	3.69±	19.39	29.12		
A	0.18*,a,	0.37*	±0.12	±1.14[a] c	*,a	
G	23.99±	4.60±	19.23	27.79		
L	0.13*,a,	1.12[a]	±0.07	±1.17 c	*,a	*,a

Data are represented as Mean ± SEM. n=6.
* Significantly different from NC at p<0.05,

a= Significantly different from DC at p<0.05, c= Significantly different from Met at p<0.05.

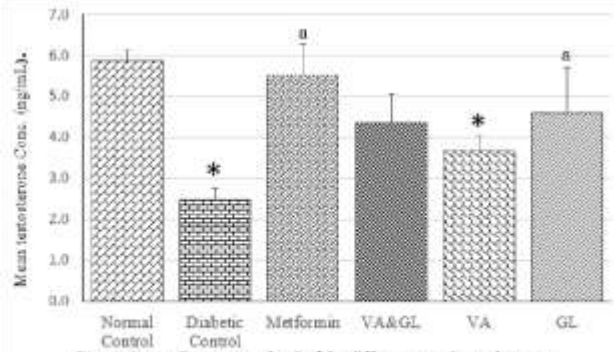

Fig. 1: Serum Testosterone level of the different experimental groups.
Data are represented as Mean ± SEM. n=6.
* Meaningfully disparate from NC at p<0.05
a=p<0.05 vs DC
VA-*Vernonia Amygdalina*, GL- *Gongronema Latifolia*

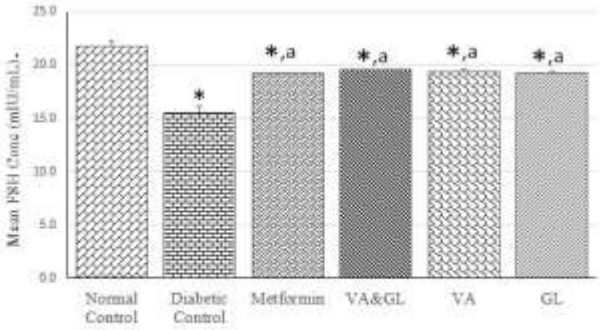

Fig 2: Serum FSH concentrations of the different experimental groups.
Values are expressed as Mean ± SEM. n=6.
* significantly different from NC at p<0.05
a=p<0.05 vs DC c=p<0.05 vs Met.
VA-*Vernonia Amygdalina*, GL- *Gongronema Latifolia*

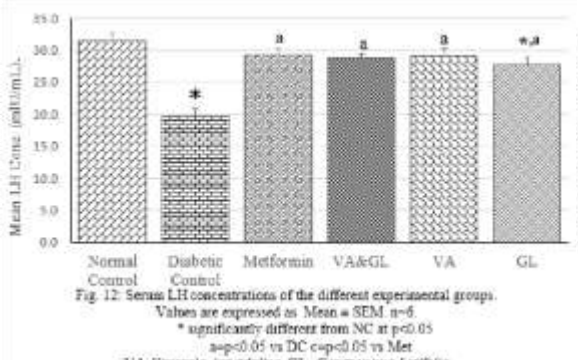

Fig. 12: Serum LH concentrations of the different experimental groups.
Values are expressed as Mean ± SEM. n=6.
* significantly different from NC at p<0.05
a=p<0.05 vs DC c=p<0.05 vs Met
VA-*Vernonia Amygdalina*, GL- *Gongronema Latifolia*

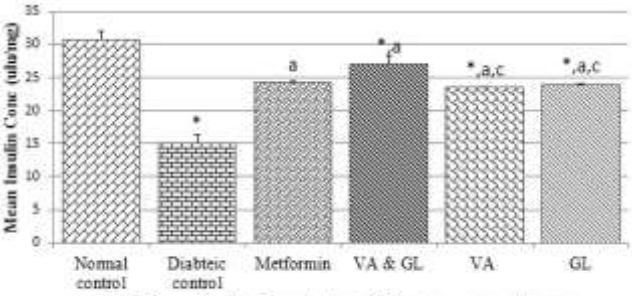

Fig.13: Serum Insulin Concentration of different experimental groups.
Values are expressed as Mean ± SEM. n=6
* Significantly different from NC at p<0.05....

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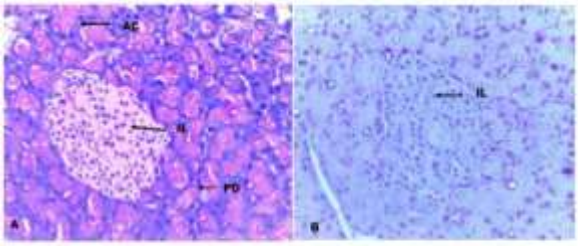

PLATE 1: Photomicrograph (X400) of pancreas of Normal Control animals, stained with (A) H & E (B) Feulgen's Reaction
The islet of Langerhans is present and well circumscribed.
The various cells in the Islet are present and prominent
The Acinar cells are present and there is a pancreatic duct observed in the specimen

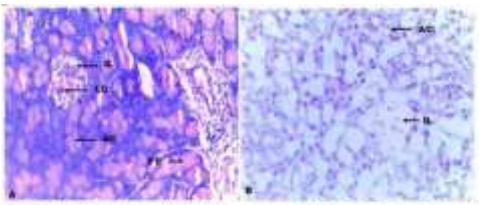

PLATE 2: Photomicrograph (X400) of pancreas of Diabetic Control animals, stained with (A) H & E (B) Feulgen's Reaction
The Islet of Langerhans appear shrunken and pyknotic
Lymphocytes (inflammatory cells) are present in the islet
Cellularity of the islet cells appear reduced and fibrous tissues are present in the islet. Acinar cells are present and appear normal
The Pancreatic Islet is shrunken and the cells are negative for Feulgen's Reaction. The acinar cells are strongly positive for Feulgen's reaction

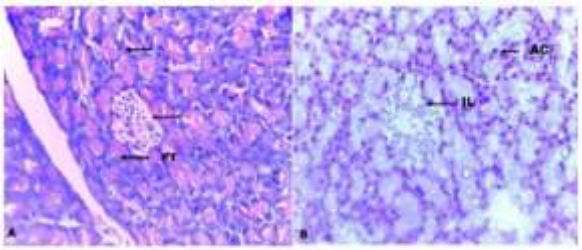

PLATE 3: Photomicrograph (X400) of Pancreas of Group C animals given 5mg/kg of Metformin, stained with (A) H & E (B) Feulgen's Reaction
The islet of Langerhans appear shrunken and degenerated. Acinar cells are present and appear normal
Most of the Islet cells are weakly positive compared to the acinar cells

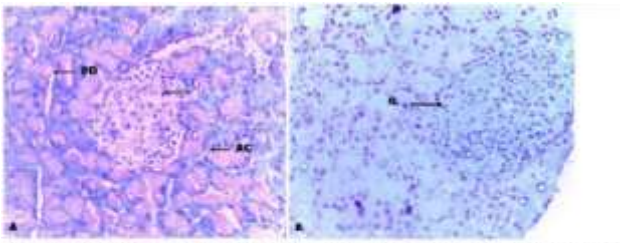

PLATE 4: Photomicrograph (X400) of Pancreas of Group D animals given 400mg/kg of combined extracts of *Gongronema latifolium* and *Vernonia amygdalina* (200mg/kg each), stained with (A) H & E (B) Feulgen's Reaction
There is a prominent islet of Langerhans pancreatic duct and normal acinar cells
There is a prominent Islet with cells that are strongly positive for Feulgen's Reaction.

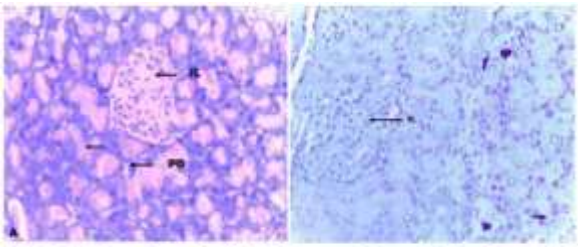

PLATE 13: Photomicrograph (X400) of Pancreas of Group F animals given 200mg/kg of *Gongronema latifolium*, stained with (A) H & E (B) Feulgen's Reaction
There are prominent pancreatic ducts, properly circumscribed islet of Langerhans and acinar cells
Most of the cells in the pancreatic Islet are strongly positive for Feulgen's Reaction

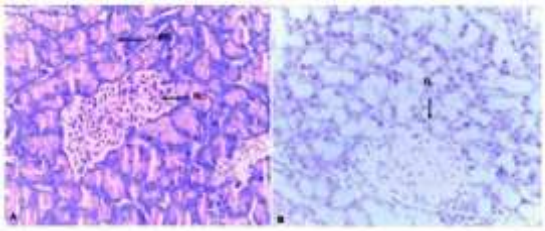

PLATE 5: Photomicrograph (X400) of Pancreas of Group E animals given 200mg/kg of *Vernonia amygdalina* extract, stained with (A) H & E (B) Feulgen's Reaction
There is a prominent islet of Langerhans, pancreatic duct and prominent acinar cells
There is a prominent Islet with few strongly positive cells interspersed with weakly positive cells.
Some of the cells in the islet are negative for Feulgen's Reaction.

Discussion

The results in this work showed that the level of the reproductive hormones in the diabetic control group was significantly reduced compared to the normal control and the groups that received the treatments. The levels of follicle stimulating hormone, luteinizing hormone, and testosterone were significantly decreased showing impairment in Leydig cell function. According to Rehman (2001), impaired Leydig cell function causing declined testosterone level has been found in diabetic men. Ballester *et al* (2004) posits that insulin dependent diabetes as was induced in this study is associated with decreased Leydig cell physiology and impaired testosterone production due to lack of the stimulatory effect of insulin on Leydig cells and an insulin contingent reduction in FSH and LH. Insulin level was also seen to be significantly reduced in the diabetic control group compared to the normal control showing damage to the insulin secreting beta cells by streptozocin as confirmed in the photomicrograph of the diabetic group. Hyperglycaemia which was observed in the diabetic control animals in this study, especially when sustained is associated with multiple complications such as male reproductive

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malfunction and impotency (Jain and Jangir, 2014). Diabetes affects male reproductive functions at multiple levels including variation in sperm quality, altered spermatogenesis, morphological changes in testes, altered sugar metabolism in Sertoli-blood-testis wall, reduced testosterone, ejaculatory dysfunction and reduced libido (La vignera et al., 2013; Alves et al., 2013). DM induced adverse effects on male reproductive functions might be interposed via hormonal modification in the hypothalamus-pituitary-gonadal axis or via straight interactions of insulin with the testes and spermatocytes as both the testes and spermatocytes produce insulin (Schoeller et al., 2012). Diabetic men and knockout mice are said to have remarkably inhibited spermatogenesis, elevated germ cell depletion and Sertoli cell vacuolization, suggesting that insulin may have a major role-play in spermatogenesis (Baccetti et al., 2002; Bruning et al., 2000).

In the groups that received the plant extracts (combined extracts of VA&GL) and metformin in this study, there was a significant increase in the hormone levels. Treatment with the plant extracts also increased insulin level significantly compared to the diabetic control. The improvements in the hormone levels may have been due to the stimulatory effect of the extracts on insulin release, their possible regenerative effect on the testicular cells and the pancreatic beta cells and the antidiabetic and antioxidant activity of the phytoconstituents present in the plant extracts. Meftormin on the other hand may have caused an improvement in the levels of insulin and reproductive hormones due to its hypoglycaemic effect. Its mechanism of action is increasing tissue sensitivity to insulin through its effect on insulin receptor expression and tyrosine kinase activity, and enhancing glucose uptake by the tissues (Gunton e*t al*., 2003). Metformin also reduces blood glucose by reducing hepatic glucose output primarily by impairing gluconeogenesis (Natali and Ferrannini, 2006)

SUMMARY AND CONCLUSION

This study elucidated the effect of methanolic extracts of the leaves of VA and GL individually and in combination and metformin on the serum glucose levels, insulin concentration and levels of reproductive hormones of STZ induced diabetic male Wistar rats.

Results revealed a significant weight loss, hyperglycaemia, DNA damage in pancreatic islet cells, and decreased levels of reproductive hormones in the diabetic control group indicating damage to the pancreatic islet cells DNA by STZ and hyperglycaemia.These effects were reversed on administration of the plant extracts and treatment with metformin. In the groups that received the combined extracts, the reversal of diabetic insults on the histology of the pancreas was close to the normal control. This effect is suggested to be due to the presence of secondary metabolites (phytochemicals) in the plants. The combined extracts of VA and GL demonstrated a synergistic action in the reversal of the effect of diabetes on the tissues and on all the parameters that were investigated.

In conclusion therefore, methanolic extracts of VA and GL especially when used in combination can be actionable in the management of diabetes mellitus and for the improvement of infertility associated with diabetes mellitus.

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