Original Research Article

Effects of the Interation of Metformin and *Vernonia amygdalina* (Bitter Leaf) On Steptozotocin-Induced Diabetic Rats.

Abstract

The effects of *Vernonia amygdalina* and metformin in lowering glucose in streptozotocin-induced diabetic rats were evaluated. A total of 120 wistar albino males and females rats weighing approximately 200g were used for the study. Diabetes was induced in the rats using 50mg/kg of streptozotocin and it was confirmed by checking the glucose levels of the rats. Rats with glucose level greater than 10mmol/L were considered diabetic. The extract, metformin and a combination of the extract and metformin were given orally to different groups of diabetic rats daily for 10 weeks. Four rats were sacrificed every 2 weeks and blood samples were collected from all the groups to estimate glucose, total protein and liver enzymes. The data obtained were compared using analysis of variance (ANOVA) and the difference between groups were established using Dunnets. The extract and metformin produced significant ($P<0.05$) decrease in plasma glucose concentrations in the diabetic rats. There was also a reduction in the plasma glucose of the rats that received a combination of the extract and metformin. The decrease in the blood glucose concentrations of the diabetic rats following the administration of the extract suggests that it possesses hypoglycemic effects on streptozotocin-induced diabetic rats. The presence of flavonoids, saponins and other phytochemicals in the extract must have acted to potentiate the hypoglycemic role of the extract.

Keywords: *Diabetes mellitus, metformin, streptozotocin*

Introduction

Diabetes mellitus describes a disorder of metabolism defined by hyperglycaemia with improper metabolism of carbohydrate resulting from abnormality in insulin production [1]. The abnormalities in insulin secretion or action are as a result of hyposecretion of insulin or insensitivity to the insulin produced. Diabetes Mellitus is defined by the World Health Organisation as a Fasting Plasma Glucose above 7.7mmol/L and a two hours postprandial plasma Glucose of 11.1mmol/L. Diabetes mellitus is a long term disease resulting from ineffectiveness of the insulin produced or by deficiency in production of insulin (which could be inherited or acquired) by the pancreas. [2]. The World Health Organization reported that Diabetes kills over one million people yearly. It also predicts life expectancy to reduce throughout the world for the first time in over two hundred years because of diabetes [3].

The use of Vernonia amygdalina (bitter leaf) in the management of diabetes mellitus is very common among traditional medicine practitioners. Fluids from fresh leaves of V. amygdalina exhibit hypoglycaemic effects. There are many bioactive constitutes present in the leaves which are responsible for these effects. The hypoglycaemic activity of the extract is due to the presence of phytochemicals such as steroid, glycoside and lactones like vernodalin.

Metformin acts by lowering glucose production by the liver and enhancing glucose uptake by body tissues. This is useful in people who are overweight because it is not associated with weight gain. It is generally well tolerated and common side effects include diarrhoea, nausea, and abdominal pain. Metformin is contraindicated in people with any condition that could increase the risk of lactic acidosis, including kidney disorders, lung disease and liver disease [4]. The potential side effect of metformin use is lactic acidosis (metformin-associated lactic acidosis).

Materials and Method

The materials that were used in this research include: glucometer (Accuchek Active by Roche), spectrophotometer,centrifuge, Randox reagent for AST, ALT, ALK and glucose strips bought from I T Johnson medical equipments limited Port-Harcourt; Streptocotozin and metformin from Winposh Pharmacy Limited Akpajo Port-Harcourt.

Animals

Albino rats were purchased from Biochemistry Department Animal House in University of Port-Harcourt. A total of one hundred and twenty (120) albino rats weighing between 180-200g body weights were used. The rats were separated into four groups consisting of twenty four rats. Each group were kept in different cages at normal and standard laboratory conditions of temperature (28 ± 2^{o}C) and relative humidity (46 ± 6%). Principle of Laboratory Animal Care were followed during the experimental and the rats gained free access to water and food Before the commencements of the experiment, the rats were allowed to acclimatize to the environment for a period of seven days.

Plant sample

Bitter leaves were purchased from Elijiji market in Woji area of Port Harcourt, Rivers state Nigeria. They were identified in the plant science department of the University of Port-Harcourt.

The rats were deprived from food and water for twelve hours and blood sample were collected from the tail to test for fasting blood glucose using a glucometer. The rats were then induced with 50mg/kg streptozotocin interperitoneally to make them diabetic. The diabetic rats were given the bitter leaf extract and metformin by oral gavage every morning before food for 10 weeks. Four rats from each group were sacrificed every fourteen days and blood samples were obtained to check for AST, ALT, ALP and glucose. All ethical issues relating to handling and storage were observed.

Extract preparation

The leaves were properly washed without squeezing then air dried at room temperature. The dried leaves were ground into powder using a manual blender. The LD_{50} of the VA extract has been reported to be 1265 ± 56mg/kg [14]). Dose of 200mg/kg was selected and prepared by dissolving 20mg of the powdered bitter leaf in 1ml of distilled water. The mixture was allowed to stand for 24 hours with occasional shaking. The mixture was then filtered and the filtrate stored in bottle.

Induction of Diabetes using streptozotocin

The rats were given 50mg/kg streptozotocin intraperitoneally to make them diabetic (this was confirmed by measuring the glucose level after 48hours). Each rat was restrained and turned over so that the abdomen was exposed. The injection was then made on the left quadrant of the abdomen avoiding the visceral organs.

After administration of streptozotocin the animals were restrained physically and blood samples were collected by tail venipunture after 48 hours and glucose concentrations were determined using a glucometer.

Preparation of Metformin (standard drug) solution

A 500mg tablet of Metformin was ground to powder and 100mg was weighed out. Dosage was prepared by dissolving the powder in a solution of 1ml of 0.9% normal saline.

Experimental Design

The study were divided into five groups with each group consisting of 24 rats

Group 1:Diabetic rats treated with 2ml of 20mg/ml of extract

Group 2:Diabetic rats treated with 2ml of 50mg/ml of metformin

Group 3:Diabetic rats treated with 1ml of 20mg/ml of extract and 1ml of 50mg/kg metformin

Group 4:Normal control rats treated with 2ml of distilled water

Group 5:Diabetic control rats treated with 2ml distilled water.

The experiment lasted for 10 weeks. However, four animals from each groups were sacrificed every two weeks by anesthetizing with chloroform. Blood samples were collected by cardiac puncture into plain and floride oxalate bottles for the determination of glucose, total protein and AST, ALT, and ALP.

METHODOLOGY

Estimation of glucose concentration

Method of estimation of glucose concentration using glucometer

The principle is based on electrochemical technology using electrochemical strips. The strips contain an enzyme, glucose dehyrogenase and a chemical, ferricyanide. The glucose dehyrogenase reacts with glucose in the blood to form glucoronic acid. The glucoronic acid form will react with ferricyanide to form ferrocyanide. The glucometer then will produce an electric current which is able to read the ferrocyanide and determine the concentration of glucose in the blood which is then displayed on the screen of the glucometer, [5].

Method of glucose estimation in plasma using spectrophometer (Glucose-oxidase method)

Principle – Glucose oxidase catalyses the oxidation of glucose to produce hydrogen peroxide and glucoronic acid. The hydrogen peroxide in the presence of enzyme peroxidase is broken down and the oxygen given off reacts with 4-aminophenazone and phenol to give a pink colour [6].

Estimation of AST

Method of AST estimation in plasma using spectrophometer (Reitman and Frankel method)

Principle- Aspartate aminoteransferase catalyses the transfer of amino acid group from aspartate to ketoglutarate, to form oxaloacetate and glutamate. The oxaloacetate so formed reacts with 2,4 dinitrophenyl hydrazine to form 2, 4, dinitrophenylhydrazone which in alkane pH of 7.5 is red brown[6].

Estimation of ALT

Method of ALT estimation in plasma using spectrophometer (Reitman and Frankel method)

Principle- Alanine aminotransferase catalyses the transfer of amino acid group from L-alanine to L-glutamate to form ketoglutarate and pyruvate. The ketoglutarate so formed reacts with 2,4 dinitrophenyl hydrazine to form 2, 4, dinitrophenylhydrazone which in alkaline pH of 7.5 is red brown [6].

Estimation of ALP

Method of ALP estimation in plasma using spectrophometer.

Principle- Alkaline phosphatise hydrolyses disodium phenyl phosphate to release phenol. The amount of phenol released is measured by the absorbance of the red colour it assumes in alkaline solution [6].

STATISTICAL ANALYSIS

The results were presented as Mean±SD. Statistical comparism between groups was done by one way Analysis of Variance (ANOVA). Significant differences between mean values of different groups were determined by one way Analysis of Variance (ANOVA) and Dunnets post hoc tests. Data were analysed by SPSS software version 20. Differences were considered significant at $p < 0.05$.

Results

Table 1: Comparison of Glucose Levels (Mmol/L) In Extract-treated, Metformin-treated, Metformin plus Extract-treated, Normal control and Diabetic control groups over a period of 10 weeks

	Start	2nd week	4th week	6th week	8th week	10th week
Grp 1 (20mg/ml BLE)	15.60±8.44[a]	15.13±6.33[a]	12.40±6.63[a]	8.60±1.57[b]	7.58±0.09[b]	7.55±1.38[a]
Grp 2 (50mg/kg MET)	14.40±3.20[a]	6.88±0.75[a]	6.05±0.70[a]	5.35±0.70[a]	5.75±0.96[a]	5.20±1.01[a]
Grp 3 (20mg/ml BLE+ MET)	16.80±6.41[a]	6.75±1.11[a]	10.25±7.00[a]	6.50±1.27[a]	6.53±0.55[a]	6.28±2.21[a]
Grp 4 (WATER)	5.88±0.81[a]	5.48±0.41[b]	5.48±0.17[b]	5.58±0.37[b]	5.45±0.26[b]	5.60±2.01[b]
Grp 5(WATER)	19.78±6.83	19.75±6.24	26.33±0.56	25.35±1.00	24.38±0.48	12.53±4.43
P-Value	0.145	0.003	<0.0001	<0.0001	<0.0001	0.544

KEY:BLE- Bitter leaf extract, MET- Metformin, [a]- Not significant, [b]-Significant.

Table 1 showed the comparison of glucose levels in the rats. The results are expressed as mean ± S D. The data were analysed using ANOVA followed by the Dunnet's test. There was significant reduction in plasma glucose of all the rats in the various groups as compared to the diabetic control group at $p<0.05$(group 5) except for the normal control group (group 4).

Table 2 Comparison of AST (U/L) Levels In Extract-treated, Metformin-treated, Metformin plus Extract-treated, Normal control and Diabetic control groups over a period of 10 weeks

	Start	2nd week	4th week	6th week	8th week	10th week
Grp 1 (20mg/ml BLE)	24.50±4.50[a]	27.00±3.72[a]	34.50±2.22[a]	30.75±2.99[a]	34.00±4.97[a]	34.25±3.86[a]
Grp 2 (50mg/kg MET)	27.75±11.85[a]	30.75±5.65[a]	35.75±3.86[a]	33.00±6.06[a]	29.25±7.18[a]	26.50±1.73[a]
Grp 3 (20mg/ml BLE+ MET)	25.50±11.45[a]	26.25±1.26[a]	39.50±3.00[a]	36.25±3.50[a]	34.00±7.44[a]	27.75±3.86[a]
Grp 4 (WATER)	31.25±1.26[a]	31.25±1.50[a]	29.75±0.50[a]	45.00±3.56[a]	29.25±0.96[a]	30.50±2.08[a]
Grp 5(WATER)	38.00±1.41	39.50±2.08	38.00±5.66	32.25±7.82	45.50±1.91	45.00±0.82
P-Value	0.313	0.001	0.001	<0.001	0.001	<0.0001

KEY: BLE- Bitter leaf extract, MET- Metformin, [a] - Not significant.

Table 2 showed the comparison of AST levels in the rats. The results are expressed as mean ± S D. The data were analysed using ANOVA followed by the Dunnet's test. There was no significant effect in AST levels of all the rats in the various groups as compared to the diabetic control group at $p<0.05$(group 5).

Table 3 Comparison of ALT (U/L) Levels In Extract-treated, Metformin-treated, Metformin plus Extract-treated, Normal control and Diabetic control groups over a period of 10 weeks

	Start	2nd week	4th week	6th week	8th week	10th week
Grp 1 (20mg/ml BLE)	25.25±5.12[a]	11.25±2.99[a]	14.25±3.86[a]	13.25±3.59[a]	11.75±5.91[a]	14.25±3.86[a]
Grp 2 (50mg/kg MET)	21.00±7.39[a]	10.25±1.50[a]	15.75±5.56[a]	13.75±4.19[a]	14.25±2.63[a]	12.50±2.38[a]
Grp 3 (20mg/ml BLE+ MET)	19.50±7.77[a]	10.75±1.50[a]	11.75±2.36[a]	12.00±0.82[a]	12.50±1.73[a]	12.00±2.16[a]
Grp 4 (WATER)	11.25±4.03[a]	21.75±6.13[a]	37.75±5.32[a]	31.25±17.59[a]	49.50±14.64[a]	48.75±1.00[a]
Grp 5(WATER)	17.25±0.96	17.50±1.91	12.00±1.63	11.75±2.36	10.75±0.96	10.50±13.64
P-Value	0.509	0.001	<0.0001	0.006	<0.0001	<0.0001

KEY: BLE- Bitter leaf extract, MET- Metformin, [a] - Not significant,

Table 3 showed the comparison of ALT levels in the rats. The results are expressed as mean ± S D. The data were analysed using ANOVA followed by the Dunnet's test. There was no significant

effect in ALT levels of all the rats in the various groups as compared to the diabetic control group at $p<0.05$(group 5)..

Table 4 Comparison of ALP (U/L) Levels In Extract-treated, Metformin-treated, Metformin plus Extract-treated, Normal control and Diabetic control groups over a period of 10 weeks

	Start	2nd week	4th week	6th week	8th week	10th week
Grp 1 (20mg/ml BLE)	39.25±6.18 [a]	35.00±6.22 [a]	41.25±2.99 [a]	40.00±5.10[a]	46.75±4.86[a]	47.25±4.43[a]
Grp 2 (50mg/kg MET)	52.00±5.72 [a]	49.00±7.75 [a]	53.25±10.90 [a]	51.25±11.32[a]	42.75±5.44[a]	43.25±2.22[a]
Grp 3 (20mg/ml BLE+ MET)	43.50±4.34 [a]	40.00±1.63 [a]	46.50±2.52 [a]	43.25±3.95 [a]	49.75±13.23[a]	48.25±16.01[a]
Grp 4 (WATER)	48.00±11.83 [a]	52.25±9.74 [a]	65.75±2.50 [a]	68.50±1.91 [a]	68.25±3.30 [a]	71.25±2.22[a]
Grp 5(WATER)	38.25±2.75	38.75±1.50	49.75±12.45	48.25±10.44	49.25±17.46	48.25±12.45
P-Value	0.072	<0.0001	0.013	0.002	0.026	0.003

KEY: BLE- Bitter leaf extract, MET- Metformin, [a] - Not significant.

Table 4 showed the comparison of ALP levels in the rats. The results are expressed as mean ± S D. The data were analysed using ANOVA followed by the Dunnet's test. There was no significant effect in ALP levels of all the rats in the various groups as compared to the diabetic control group at $p<0.05$ (group 5).

Table 5: Comparison of Protein (g/L) Levels In Extract-treated, Metformin-treated, Metformin plus Extract-treated, Normal control and Diabetic control groups over a period of 10 weeks

	Start	2nd Week	4th Week	6th Week	8th Week	10th Week
Grp 1 (20mg/ml BLE)	53.25±5.06 [a]	53.25±12.69[a]	66.00±4.55 [a]	68.00±5.42 [a]	70.25±12.15 [a]	66.50±8.58 [a]
Grp 2 (50mg/kg MET)	57.00±4.69 [a]	52.00±0.01 [a]	64.50±6.40 [a]	60.25±6.85 [a]	63.50±4.73 [a]	67.50±2.38 [a]
Grp 3 (20mg/ml BLE+ MET)	59.75±3.86 [a]	54.00±2.71 [a]	58.50±7.72 [a]	55.75±4.92 [a]	69.75±4.79 [a]	68.75±2.63 [a]
Grp 4 (WATER)	66.75±6.90 [a]	71.25±5.38 [a]	80.00±10.36 [a]	82.75±3.86 [a]	80.75±4.57 [a]	88.00±3.65 [a]
Grp 5(WATER)	62.50±8.58	63.75±6.08	65.50±8.79	62.50±4.43	65.25±2.63	66.75±3.30
P-value	0.158	0.002	0.0005	<0.0001	0.039	<0.0001

KEY: BLE- Bitter leaf extract, MET- Metformin, [a] - Not significan.

Table 5 showed the comparison of TP levels in the rats. The results are expressed as mean ± S D. The data were analysed using ANOVA followed by the Dunnet's test. There was no significant effect in TP levels of all the rats in the various groups as compared to the diabetic control group at $p<0.05$.

Discussion

The bitter leaf extract at concentration of 20mg/ml was able to cause a significant reduction in the glucose concentrations of the diabetic rats. This corroborates the result of other researchers, who had also demonstrated that the extract from the plant has hypoglycemic properties [7] [8]. These properties are attributable to the phytochemicals present in the plant. The phytochemicals include Terpenoids, Alkaloids, Phenol, Saponin, Tannin and Flavonoids [9]. Phenols are reported to inhibit actions of alpha amylase, sucrase, and sodium glucose transporter of the intestinal brush border cells, thereby reducing glucose levels. Saponins also lowers blood glucose by insulin response restoration, Alpha- glucosidase activity inhibition, inhibition of gluconeogenesis, disaccharide activity inhibition and so on [4]. Flavonoids present in the extract causes proliferation and secretion of more insulin which may also contributed to the lowering of the glucose level [10]. The extract may also have some insulin-like substances, and induction of regenerative stimulus in diabetic stage which triggers pancreatic regenerative processes, thereby restoring functional activities of the pancreas [11]. There was a great decrease in the level of glucose of the diabetic rats receiving metformin alone throughout the duration of the experiment. The rate of decrease might probably lead to hypoglycaemia if the duration of the experiment is extended. This collaborates with some report that long term use of metformin can result to hypoglycaemia. The mechanism by which this can occur include; reduction in hepatic glucose production, decreased glucose absorption and poor oral intake [12]. The group receiving the combination of the extract and metformin shows a significant reduction in the level of glucose by the 2^{nd} week followed by an increase by the 4^{th} week. The increase may be probably due to enzymatic induction. By the 6^{th} week the glucose level begin to reduced again till the 10^{th} week. But the rate of reduction is not like that of metformin alone. This implies that reducing the dose of metformin and augmenting it with the extract also reduces the glucose concentration significantly indicating that the combination therapy is also effective and the side effects of metformin can be reduced.

The hypoglycemic effect of the combination of the extract and metformin implies that their antidiabetic activities are addictive and this suggests that they are both acting through the same mechanism. According to [4], metformin acts primarily at the liver by reducing glucose production and secondarily, by increasing glucose uptake in the peripheral tissues especially the muscle. Metformin also reduces the concentration of glucose in the blood by decreasing hepatic gluconeogenesis, that is, synthesis of glucose by the liver. This may explain why metformin alone and in combination significantly reduced the blood glucose. Reducing the dose of metformin and augmenting it with the extract also reduces the glucose concentration significantly. This shows that the combination therapy is also effective and the side effects of metformin can be reduced. In addition, there was slight increase in AST level of all the diabetic rats receiving the extract from the beginning to the 4^{th} week but the values started reducing again by the 6^{th} week. The ALT levels show slight increase by the 2^{nd} week but it increased again by the 4^{th} week same as the ALP and total protein. The increases and decreases observed in the values of the liver enzymes and total protein were still in the reference range throughout the period of the experiment. This may be because of the hepatoprotective ability of the bitter leaf extract due to its antioxidant property.

This property is attributable to flavonoids present in the extract. Flavonoids are reported to exhibit antioxidant activity and are effective scavengers of superoxide anions [13].

Conclusion

This research has demonstrated that the extract of bitter leaf has antidiabetic effects. The use of a combination of the extract and metformin is also effective and safe for the management of diabetes. The use of bitter leaf extract is advised due to lot of side effects associated with the use of hypoglycaemic agents like metformin.

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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