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Full length Research Article

Anti-Diabetic Activities of the Hydromethanolic Leaf Extract of *Rauvolfia vomitoria*.

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Summary: *Rauvolfia vomitoria* (African snake root) is an herbal shrub which has been reported to possessing strong anti-diabetic action; however, the method(s) by which this plant effects its reported anti-diabetic action is poorly understood. Hence, this research through in vitro and in vivo studies investigated the methods (mechanisms of action) through which this plant effects its reported anti-diabetic action. In the in vitro study, the effect of the hydromethanolic leaf extract of the plant on alpha amylase and alpha glucosidase enzymes were investigated in comparison with acarbose; while in the in vivo study, the effect of the extract on blood sugar and plasma insulin levels of normal and streptozotocin-induced diabetic albino rats were investigated in comparison with glyburide. Results from the in vitro study showed the percentage inhibition of alpha amylase by the extract (100 mg/ml) to be 62.28 (4.73) with an IC_{50} values of 74.35 mg/ml, while acarbose had a percentage inhibition and IC_{50} values of 72.81 (2.52) and 66.05µg/ml, respectively. The percentage inhibition of alpha glucosidase by the extract (100 mg/ml) was 79.63 (4.09) and an IC50 values of 58.85 mg/ml, while acarbose had a percentage inhibition and IC_{50} values of 82.11 (1.84) and 56.79µg/ml, respectively. From the in vivo study, the result showed that the extract caused a dose and treatment-duration dependent significant increases ($P<0.05$) in the plasma insulin levels of streptozotocin-induced diabetic rats in a manner comparable to glyburide. These results showed that *Rauvolfia vomitoria* leaf effects its anti-diabetic actions via two separate mechanisms; the plasma insulin increasing mechanism and the alpha amylase and alpha glucosidase inhibitory mechanism.

Keywords*: Rauvolfia vomitoria leaf extract, alpha amylase, alpha glucosidase, plasma insulin, acarbose, glyburide*

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INTRODUCTION

Diabetes mellitus is a health condition distinguished by severely elevated plasma sugar level ensuing from either a dysfunction of the beta cells of the islets of Langerhans (pancreatic islets) which often results to insufficient insulin secretion (type-1) or cells of the body not responding to insulin (type-2) (N'doua *et al*., 2016; Akpojotor and Ebomoyi, 2021a).

The prevalence of diabetes is constantly increasing globally. In 1980, it was 108 million people that was estimated to have diabetes worldwide (World Health Organization, 2016). By 2013, the figure had increased to 382 million (Shi and Hu, 2014) and as at 2019, the figure has further increased to 463 million (Saeedi *et al*., 2019). More increases have been forecasted (World Health Organization, 2008; Piero *et al*., 2014; Saeedi *et al*., 2019). This health condition has become a great source of concern due to its increasing prevalence and the inability of orthodox drugs in offering absolute cure to it (Okpuzor *et al*., 2009; Kumari *et al*., 2013; Akpojotor and Ebomoyi, 2021a).

The use of medicinal plants (herbs) as therapy for management of diabetes mellitus and other ailments is an age long practice (Mbaka *et al*., 2010; Akpojotor and Kagbo, 2016; Udia *et al*., 2016). The ailment-specific pharmacological basis (such as active phytoconstituents, mechanisms of action and possible side effects) of some of these medicinal plants and their formulations have been elucidated by scientific investigations (Udia *et al*., 2016), while many others including *Rauvolfia vomitoria* are yet to be elucidated.

Rauvolfia vomitoria commonly known as African snake root is a plant species in genus *Rauvolfia* under the apocynaceae family (Ajayi, 2021). It mainly grows wildly in different forests across the globe especially in tropical Africa, South America and Asia (Amole, 2003; Ogbe *et al*., 2009; Akpojotor and Ebomoyi, 2021a). Various studies have attributed a range of medicinal actions to it including treatment of cancer (Yu *et al*., 2013), hypertension (Amole, 2003; Ezejindu *et al*., 2013), convulsion (Olatokunboh *et al*., 2009), mental derangement (Bisong *et al*., 2010), diabetes (Campbell-Tofte *et al*., 2011), etc. On its diabetic medicinal action, available literatures mainly report its plasma sugar lowering (hypoglycemic) ability (Compbell-Tofte *et al*., 2011; N'doua *et al*., 2015; N'doua *et al*., 2016), while vital information such as the biochemical constituents through which the plant effect this action as well as the method (mechanism of action) are still lacking. This

therefore, necessitated this investigation which was designed to examine the mechanisms through which *Rauvolfia vomitoria* leaf effects its anti-diabetic action by examining its effects on plasma insulin, alpha amylase and alpha glucosidase levels in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

This study was carried out in two phases (an *in vitro* and an *in vivo* phase). In the *in vitro* phase, the effect of the hydromethanolic leaf extract of the plant on alpha amylase and alpha glucosidase enzymes were investigated in comparison with acarbose, a standard alpha amylase and alpha glucosidase inhibitory drug. In the *in vivo* phase, the action of the plant on plasma insulin was investigated in comparison with glyburide, a standard anti-diabetic drug that effects its anti-diabetic action by enhancing insulin secretion. Streptozotocin-induced diabetic male adult albino rats was used as models.

Research Protocol Approval: Approval for research and animal handling for this research investigation was granted by the Research Ethics Committee in the College of Medical Sciences of the University of Benin, Benin City, Nigeria. App. No.: CMS/REC/2021/159.

Chemicals and Drugs: Insulin and Glucagon immunoassay kits (Calbiotech, Inc. 1935 Cordell Ct., CA, USA); Streptozotocin, *Saccharomyces cerevisiae* and paranitrophenyl-glucopyranoside (Sigma-Adrich Co., St Louis, USA.); Purified porcine pancreatic *α*-amylase (ICN Biochemicals, Ohio, USA) were acquired from BioRapid Diagnostic Nig. Ltd., Abuja. Accu‑Chek Active blood glucose meter and Accu‑Chek Active blood sugar testing strips; glyburide and acarbose were obtained from Green House Pharmacy, at the University of Port Harcourt Teaching Hospital, Rivers state, Nigeria.

Plant Sample (Collection and Identification): *Rauvolfia vomitoria* leaves were collected from open farmlands in Orhuwhorun village in the South-South region of Nigeria. The plant sample was authenticated by E. Nwosu at the Ecoland Herbarium (EH) Unit, University of Port Harcourt Choba, Rivers State, with EH. ID. number EH – P – 051.

Extraction of Active Components from Plant sample: Collected leaves of *Rauvolfia vomitoria* were rinsed in clean water to rid all dirt and then air-dried in a well-ventilated room for two weeks. The dried leaves were then milled to fine powder and thereafter subjected to maceration extraction using hydromethanol (ratio of water to methanol is 1:4) as extraction solvent following the method of Akpojotor and Kagbo, 2016. In brief, the milled sample was immersed in sufficient extraction solvent. The mixture was stirred from time to time within forty-eight hours at room temperature after which Whatman No. 1 filter paper was used to separate it into two portions (a filtrate and a residue). Thereafter, the filtrate was concentrated in a vacuum at 40°C using a rotary evaporator (Searl Instruments Ltd. England) into the extract used in the experiments.

Preliminary Phytochemical Profiling of Extract: Preliminary phytochemical profiling of the extract was done following the methods of Sofowora, 2008; Ibironke and Olusola, 2013; and Amita and Shalini, 2014.

***In Vitro* Experiments**

Alpha-Amylase Enzyme inhibition test: The effect of the hydromethanolic leaf extract of the plant on alpha amylase enzyme was investigated following the method used by Chelladurai and Chinnachamy (2018), with slight modification.

In preparing the starch (substrate) solution used in this experiment, 1 gram of starch from potato was dissolved in 10mls of distilled water, the mixture was boiled, cooled, and the volume increased to 100mls by the addition of more distilled water. 1 milligram of porcine pancreatic alpha amylase dissolved in 100mls of 20 mM phosphate (pH 6.9) was used as the enzyme solution. Different masses of the extract (20, 40, 60, 80 and 100 milligrams) were dissolved in dimethyl sulfoxide to prepare the different concentrations (20 to 100mg/ml) of sample solutions used. The colouring reagent was Dinitrosalicylic (DNS) solution.

Three sets of tests (test, blank and control) were conducted each in triplicate. In brief, 1ml each of the sample and enzyme solutions were mixed in a test tube and incubated under room temperature (32-33^{o}C) for 20 minutes, thereafter, 1ml of the incubated solution was mixed with 1ml of the substrate solution and incubated for 3 min at room temperature. After the 3 minutes' incubation, 1 ml of the coloring reagent was added and the mixture heated in water bath at 85^{o}C for 15 minutes after which it was diluted with distilled water (9ml) and the absorbance was recorded at 540 nm. In the blank experiment, the coloring reagent was added before the substrate solution, while the rest of the method was the same as in the test experiment. For control, all procedures were as in the test experiment except that the sample solution was replaced with 1ml of dimethyl sulfoxide. Acarbose (20 to 100µg/ml) was used as a positive (comparative) control.

The percentage inhibition of the sample was determined by mathematical calculation using the formula:

$$\% \text{ Inhibition} = \frac{[(Ac - As)/Ac] \times 100}{}$$

Ac-absorbance of control;

Where As=absorbance of sample

Alpha-Glucosidase inhibitory test: The alpha glucosidase inhibitory action of the extract was investigated using Kim *et al*. (2005) method as described by Kazeem *et al*. (2013) with slight modifications. 1.01M/ml alpha glucosidase from brewer's yeast, p-nitrophenyl glucopyranoside prepared in 20mM phosphate buffer (pH 6.9) and 0.1M sodium carbonate (Na_2CO_3) were used as enzyme, substrate and reaction-stopping solutions respectively.

In brief, 100μl of the enzyme solution was added to 50μl of the different concentrations of the extract (20-100mg/ml) and incubated for 10 minutes under room temperature. Thereafter, 50μl of the substrate solution was added and incubated for 20 minutes. After the 20 minutes' incubation, 200μl of the reaction-stopping solution was added and the alpha glucosidase activity was immediately determined by measuring the yellow-colored para-nitrophenol released from p-nitrophenyl glucopyranoside at

405 nm. For control, dimethyl sulfoxide was used in place of test solutions, while for positive controls, acarbose (20, 40, 60, 80 and 100µg/ml) was used in place of the sample extract. The alpha glucosidase inhibitory activity of the extract was calculated using the mathematical formula:

% Inhibition = [1 – Ae/Ac] × 100

Where Ae=Absorbance of Extract, and Ac=Absorbance of Control

All samples were assayed in triplicate.

***In Vivo* Studies on Rats**

Induction of Diabetes: Diabetes was induced in experimental animals (male albino rats) following the method of Rossini (1977) as used by Akpojotor and Ebomoyi (2021b). In brief, experimental animals were fasted overnight and diabetes induced via intraperitoneal administration of 55mg/kg bwt streptozotocin solution (streptozotocin dissolved in citrate buffer, pH 4.5). Following the streptozotocin administration, animals having blood sugar equal to or more than 280 mg/dl were recruited as diabetic animals for this study.

Grouping of Experimental Animals: Fifty (50) albino rats (male) weighing between 180-220g were recruited for this study. Experimental animals were grouped into five groups of ten animals each as follows; **1**-Normal control group (Non-diabetic animals)**; 2**-Diabetic control group**; 3**-250mg/kg bwt extract treated group**; 4**-500mg/kg bwt extract treated group**; 5-** 5mk/kg bwt glyburide treated group. The three treated groups (group 3-5) were treated with 250mg/kg) bwt dose of the extract, 500mg/kg bwt dose of the extract and 5mg/kg bwt dose of glyburide respectively following the method of Diehl *et al*., 2001. Administrations were done daily. In brief, a flexible cannula is attached to the syringe containing the extract or drug to be administered. The delivery end of the cannula is inserted into the mouth of the animal and gently advanced into the esophagus and towards the stomach, then the extract or drug is emptied gently into the animal.

Blood Sample Collection: After 14 and 28 days of treatment, five rats from each group were sacrificed and blood collected from each sacrificed animal for hormonal assay of insulin via cardiac puncture.

Measurement of Blood Glucose Levels: The Accu-Chek Active blood glucose meter and Accu-Chek Active test strips were used for the measurement of blood glucose levels of animals. In brief, the tip of the tail of the animal was pierced with a lancet and a drop from the blood that oozes out was applied to test strip inserted into the Accu-Chek blood glucose meter. The glucose concentration in the blood was displayed on the screen of the Accu-Chek blood glucose meter. This procedure was performed on all the animals. Blood glucose levels of all groups were measured just before commencement of treatment and thereafter, after every four days of treatment.

Biochemical Analysis: Collected blood samples from sacrificed animals were subjected to hormonal assays following the methods of Tietz (1995) as used by Akpojotor and Ebomoyi (2021b). In brief, 50µL of the different concentration of standard working solution were each pipetted into the first two test pots, while blood serum from experimental animals were pipetted into the other test pots. Then 50µL of biotinylated solution was pipetted into all the test pots and they were incubated for 45 minutes. After incubation, the test pots were drained and washed using wash buffer. After washing, 100µL of Horseradish Peroxidase Conjugate working solution was pipetted into each test pot and they were incubated for 30 minutes. After incubation, the test pots were again drained and washed. Thereafter, 90µL of substrate reagent was pipetted into each test pot and incubated for 15 minutes. After incubation, stop solution (50µL) was added to each test pot and a micro-plate reader set at 450nm was used to determined the optical density (OD) value of each test pot.

Note: The Standard Working Solution of different concentrations and the test pots (wells) were provided in the ELISA immunoassay kit. All incubations were done at room temperature.

Data Analysis: The data obtained from this study were recorded as mean ± standard error of mean (SEM) and were analyzed using ANOVA. Statistical differences between means were analyzed by applying Duncan Multiple Range Test (DMRT) for comparison with control groups at 95% ($p<0.05$) confidence levelThis study was carried out in two phases (an *in vitro* and an *in vivo* phase). In the *in vitro* phase, the effect of the hydromethanolic leaf extract of the plant on alpha amylase and alpha glucosidase enzymes were investigated in comparison with acarbose, a standard alpha amylase and alpha glucosidase inhibitory drug. In the *in vivo* phase, the action of the plant on plasma insulin was investigated in comparison with glyburide, a standard anti-diabetic drug that effects its anti-diabetic action by enhancing insulin secretion. Streptozotocin-induced diabetic male adult albino rats was used as models.

RESULTS

Preliminary Phytochemical Screening of Hydromethanolic Extract of *Rauvolfia vomitoria* Leaf: Investigation on the phytochemical constituents of Hydromethanolic extract of *Rauvolfia vomitoria* Leaf revealed that it contains some bio active phytochemical compounds as tabulated in Table I below.

Table I:
Preliminary phytochemical screening of hydromethanolic *Rauvolfia vomitoria* leaf extract

Constituents	Inference
Alkaloids	+
Flavonoids	+
Saponins	+
Tannins	+
Terpenes	+
Cardiac glycosides	+
Cyanogenic glycosides	-
Anthraquinones	-

\+ *indicates presence*
\- *Indicates absence*

Table 2:
Effect of treatment with hydromethanolic (HM) leaf extract of *Rauvolfia vomitoria (Rv)* on blood glucose level (mg/dl) of Streptozotocin-induced diabetic male wistar rats

Group	Description	Just b/4 induct.	72 hrs after induct.	2 days of treat.	6 days of treat.	10 days of treat.	14 days of treat.	18 days of treat.	22 days of treat.	26 days of treat.	28 days of treat.
1	Normal control	82.80±3.34	81.60±3.61	85.60±2.71	80.40±2.84	82.80±3.95	82.00±3.08	85.00±3.16	85.00±3.63	89.00±2.10	88.20±1.62
2	Diabetic control	89.80±2.92	339.00±6.38	326.60±7.81	315.00±7.56	302.80±4.19	299.60±7.88	303.40±1.94	299.80±2.48	304.00±2.19	300.00±0.71
3	Diabetes plus 250mg/kg of extract	91.00±3.81	328.40±8.10	326.80±9.55	304.00±7.21	268.40±14.08	223.40±2.56	201.20±3.44*	146.00±10.32*	128.40±6.95*	115.00±8.76*
4	Diabetes plus 500mg/kg of extract	86.60±2.23	324.80±10.72	280.40±11.10	241.60±4.24	185.40±6.45*	127.20±4.25*	106.20±5.45*	94.20±2.22*	89.40±1.54*	87.80±3.31*
5	Diabetes plus glyburide (5mg/kg)	86.60±2.23	318.00±6.74	279.00±6.72	244.20±3.07	179.40±5.97*	130.80±3.77*	110.20±4.80*	100.60±2.25*	96.20±1.83*	81.00±4.23*

*Values are expressed as Mean±SEM; n=5; *=Significant at p<0.05*

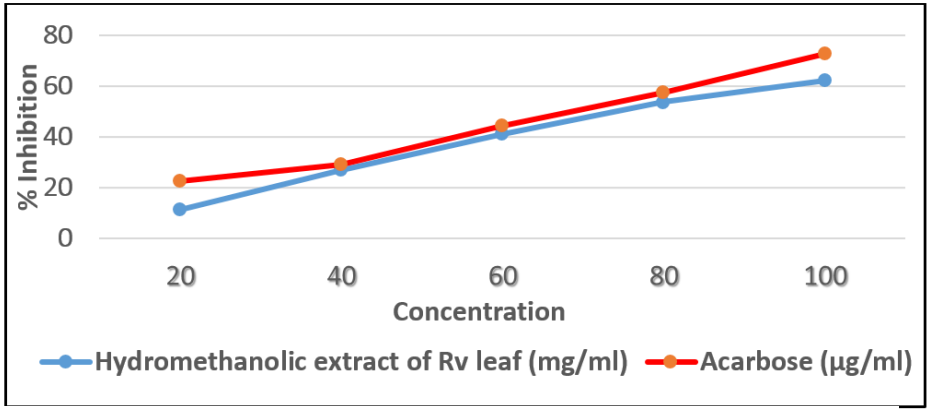

Figure I:
Effect of hydromethanolic *Rauvolfia vomitoria* leaf extract on α-amylase in comparison with acarbose

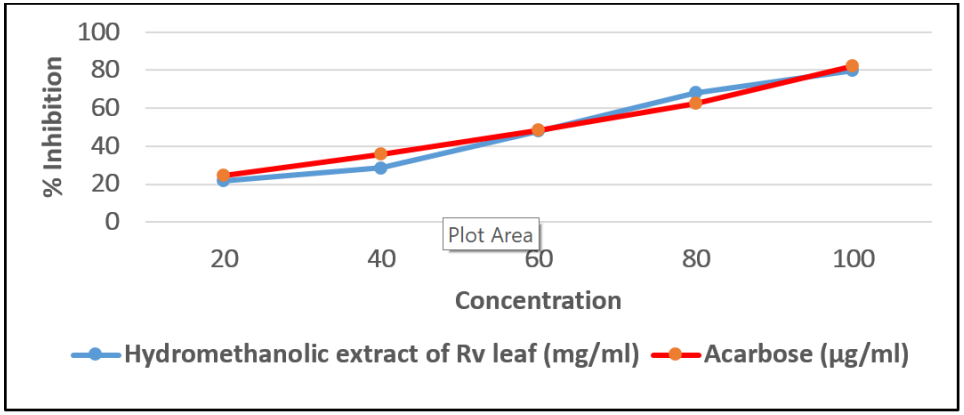

Figure 2:
Effect of hydromethanolic *Rauvolfia vomitoria* leaf extract on α-glucosidase in comparison with acarbose

***In Vitro* Study:** The in vitro study revealed that *Rauvolfia vomitoria* leaf extract inhibited alpha amylase and alpha glucosidase enzymes. As shown in figure I, different concentrations (20 - 100 mg/ml) of the extract exhibited alpha amylase inhibition in a dose dependent manner comparable to acarbose (a standard alpha amylase inhibitory drug). The IC_{50} values for the extract and acarbose were calculated as 74.35 mg/ml and 66.05µg/ml, respectively.
The result of the alpha glucosidase inhibitory action of extract (figure II) showed that the extract also has a dose dependent inhibitory effect on alpha glucosidase enzyme. The IC_{50} values for the extract and acarbose were calculated as 58.85 mg/ml and 56.79µg/ml, respectively.

The maximum percentage of inhibition for both alpha amylase and alpha glucosidase enzymes by *Rauvolfia vomitoria* leaf were obtained at a concentration of 100mg/ml and were 62.28 (4.73) % and 79.63 (4.09) %, respectively.

***In Vivo* Study:** The in vivo study revealed that treatment with *Rauvolfia vomitoria* leaf caused a dose and treatment-duration dependent effect on the insulin levels of streptozotocin-induced diabetic rats.
Table II, the tabulated result of the in vivo study indicated significant increase ($P<0.05$) in the insulin levels of streptozotocin-induced diabetic rats following treatment with 250 and 500 mg/kg bwt doses of the extract. These increases were comparable to that of the standard anti-diabetic drug (glyburide) treated group. The result also showed that the significant increase in the insulin levels of the extract treated groups were dose- and duration-dependent.

Table 2:
Effect of hydromethanolic extract of *Rauvolfia vomitoria* leaf on plasma insulin levels of Streptozotocin-induced diabetic albino rats

S/N	Description	Insulin (µU/ml) after two weeks treatment	Insulin (µU/ml) after four weeks treatment
1	Normal control group (Non-diabetic animals)	13.180 (1.550)	13.380 (0.610)
2	Diabetic control group	6.990 (0.780)	7.290 (0.720)
3	250mg/kg bwt extract treated group	9.490 (0.920)	11.190 (0.870)*
4	500mg/kg bwt extract treated group	11.800 (1.100)*	13.250 (0.720)*
5	5mk/kg bwt glyburide treated group	10.640 (0.980)*	13.980 (0.450)*

Values are expressed as Mean (SEM); n=5; *=Significant at $p<0.05$

DISCUSSION

The result of the preliminary phytochemical screening of the extract in this study is similar to those of Ojo *et al*. (2012), Okereke *et al*. (2015) and Ajayi (2021) which reported Alkaloids, flavonoids, saponins, cardiac glycosides, terpenoids, and tannins, as the bio active phytochemical compounds of *Rauvolfia vomitoria* leaf. Some of these phytochemical compounds have been shown by previous studies to possess anti-diabetic actions and can be potential natural sources for medications against diabetes. For example, Osadebe *et al.* (2010) reported that the presence of flavonoids, terpenoids and tannins in plant confer blood glucose lowering ability on it. Similarly, Etxeberria *et al*. (2012) implicated flavonoid as an inhibitor of carbohydrate digestive enzyme (α-glucosidase); Khan *et al*. (2014) listed tannins and terpenoids as the main phytoconstituents responsible for the anti-diabetic action of Chinaberry tree (*Melia azedarach*), while Poongunran *et al*. (2015) reported that flavonoids and tannins have potential inhibitory effects on alpha amylase and alpha glucosidase enzymes.
The use of medicinal plants (herbs) as therapy for management of diabetes mellitus and other ailments is an age long practice (Mbaka *et al*., 2010; Akpojotor and Kagbo, 2016; Udia *et al*., 2016). Most medicinal plants are believed to be effective in managing different ailments. But the ailment-specific pharmacological profile and actions (such as active phytoconstituents, mechanisms of action and possible side effects) of most of these medicinal plants and their formulations have not been elucidated by scientific investigations, hence our recent studies on *Rauvolfia vomitoria*.

Diabetes mellitus is a health condition distinguished by severely elevated plasma sugar level resulting from either a dysfunction of the beta cells of the pancreas which often results to insufficient insulin secretion (type-1) or cells of the body not responding to insulin (type-2). Hence, the primary focus of its treatment is to return the severely elevated blood sugar level to the normal physiological range or as close as possible. This explains why the ability to cause a decrease in the blood sugar level of an elevated blood sugar system is the first criterion a substance, drug or plant, must meet for it to be considered an anti-diabetic agent. The result of this study (table 2), showed that *Rauvolfia vomitoria* leaf has anti-diabetic potentials. Our result from this study is in line with the reports of Campbell-Tofte *et al*. (2011) and N'doua *et al*. (2016) which associated *Rauvolfia vomitoria* leaf with blood glucose lowering (hypoglycemic) activity.

The treatment of diabetes is anchored on three strategies or mechanisms which include; (1) prevention of postprandial glucose spike, (2) increasing the plasma insulin and (3) increasing body cells' sensitivity to insulin.

Alpha amylase and alpha glucosidase are the enzymes responsible for the digestion of starch (carbohydrates) in ingested food, converting them from poly-saccharides and di-saccharides into absorbable mono-saccharides (glucose and fructose) which are absorbed through the walls of the small intestine into circulation. By inhibition these enzymes, the formation of absorbable carbohydrates is inhibited and postprandial glucose spike is prevented (De Sales *et al*., 2012; Chellandurai and Chinnachamy, 2018; Alqahtani *et al*., 2020). The result from this study showed that the extract possesses strong inhibitory effects on these enzymes and therefore suggest that, one of the mechanisms through which *Rauvolfia vomitoria* leaf effects its anti-diabetic action is by alpha amylase and alpha glucosidase inhibition. This result is in line with reports of Kwon *et al*. (2006),

Kazeem *et al*. (2013) Chellandurai and Chinnachamy (2018), and Alqahtani *et al*. (2020), which associated various plants with natural alpha amylase and alpha glucosidase inhibitors.

Insulin in the body is secreted by the beta cells of the Islet of Langerhans of the pancreas. Hence, streptozotocin induces diabetes by destroying the beta cells of the pancreas (Szkudelski, 2001; Akpojotor and Ebomoyi, 2021b). Streptozotocin produces free radicals such as hydrogen peroxide and peroxynitrite which are selectively permeable to the beta cells of the pancreas (Elsner *et al*., 2000; Lenzen, 2008). Accumulation of these free radicals in the beta cell causes oxidative stress/destruction of the cell and consequently deficiency of plasma insulin (Raza *et al*., 2011; Akpojotor and Ebomoyi, 2021b). Increasing plasma insulin is one of the common approaches in the treatment of type 2 diabetes, and results obtained from this study showed that *Rauvolfia vomitoria* leaf restored physiologic insulin levels in streptozotocin-induced diabetic albino rats.

From our findings, it was concluded that *Rauvolfia vomitoria* leaf contains some bio active phytochemical compounds responsible for its anti-diabetic actions. *Rauvolfia vomitoria* leaf effects its anti-diabetic actions by minimizing the amount of glucose that is absorbed into circulation after a carbohydrate meal via its α-amylase and α-glucosidase inhibitory action; and by increasing plasma insulin levels. The inhibitory effect is more towards alpha glucosidase than alpha amylase.

This research study is the first to investigate the anti-diabetic mechanisms of action of *Rauvolfia vomitoria* leaf, it will therefore contribute significantly to the available knowledge on the anti-diabetic properties of *Rauvolfia vomitoria* and the potential for its formulation as medicine for the management of diabetes.

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