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Research Article
Antidiabetic Effect of *Viburnum foetidum* Bark in Cell Lines and Wistar Rats

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Abstract

Background and Objectives: Diabetes mellitus is a metabolic disease that is on the increase globally in recent years. *Viburnum foetidum* is a medicinal plant used in traditional medicine for the treatment of various diseases. In this study, the antidiabetic effect of *Viburnum foetidum* bark ethanolic extract (VFE) and ethanolic extract fractions (n-butanol, ethyl acetate and n-hexane) were evaluated using both *in vitro* (RIN-5F and HepG2 cells) and *in vivo* (glucose loaded [10 g kg^{-1}] and streptozotocin [60 mg kg^{-1}] induced diabetic Wistar rats) models. **Materials and Methods:** In the *in vivo* studies, 100, 200 and 400 mg kg^{-1} *Viburnum foetidum* bark ethanolic extract and 200 mg kg^{-1} of each extract fractions were administered to streptozotocin (60 mg kg^{-1}) induced diabetic Wistar rats. Non toxic mass value of *Viburnum foetidum* extract was used in the *in vitro* (0.625, 1.25 and 2.5 µg/100 µL) and *in vivo* (100, 200 and 400 mg kg^{-1}) studies. **Results:** *Viburnum foetidum* extract and extract fractions increased RIN-5F insulin secretion and HepG2 glucose uptake when compared with untreated cells. Whereas, in 21 days treated diabetic rats, the extracts caused a decrease in fasting blood glucose level, increase in serum insulin and serum α-amylase level when compared with the untreated rats. The ethyl acetate extract fraction at 200 mg kg^{-1} exerted a more increase in RIN-5F cells insulin secretion, HepG2 cells glucose uptake, serum insulin and serum α-amylase level than other extract fractions. **Conclusion:** *Viburnum foetidum* bark exerts its antidiabetic effect by enhancing insulin secretion by pancreatic cells, increasing glucose uptake by liver cells and enhancing the pancreas functionality thereby increasing serum insulin and serum α-amylase level.

Key words: *Viburnum foetidum*, diabetes, RIN-5F cells, HepG2 cells, Wistar rats

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that occurs as a result of lack of insulin production by the pancreas (Type 1 diabetes), resistance of cells to insulin (Type 2 diabetes) and also due to insufficient production of insulin by the pancreas (Type 2 diabetes)[1]. Recent reports documented that the disease is on the increase in the last few decades, from 108 million in 1980 to 422 million[2] in 2014. There are drugs with different mechanism of action for the treatment of diabetes. These drugs include insulin sensitizers (thiazolidinediones), insulin secretagogues (sulfonylureas) and inhibitors of hepatic glucose production (metformin)[3]. There are reports that these antidiabetic drugs have some side effects which include increase/decrease in weight, hypoglycemia, cardiac failure, genitourinary tract infection, gastro intestinal disorder, nausea and vomiting[3,4]. These reports have led to the recent increase in research on the possible antidiabetic effect of medicinal plants with little or no side effect. Previous studies carried out on medicinal plants showed that all or some parts of plants have medicinal potentials which include anti-inflammatory, analgesic[5], anti-malaria[6], anti-hypertensive[7] and anti-diabetic activities[8]. *Viburnum foetidum* Wall (Adoxaceae) a shrub commonly known as stinking viburnum and viburni in India is also found in China, Bhutan, Burma and north east India. In traditional medicine, all parts of the plant are reported to have astringent properties, the aerial parts are reported to have hypothermic and cardiovascular activities whereas the leaves extract is used for the treatment of menorrhagia[9].

In this study, the antidiabetic mechanism of *Viburnum foetidum* bark ethanolic extract and extract fractions (n-butanol, ethyl acetate and n-hexane) was evaluated by investigating the effect of the extracts on insulin secretion by RIN-5F pancreatic cells, glucose uptake by HepG2 liver cells, reduction in fasting blood glucose level, increase in serum insulin and serum α-amylase levels in streptozotocin-induced diabetic Wistar rats.

MATERIALS AND METHODS

This research study was carried out in 4 months (September, 2018 to January, 2019) at the School of Biotechnology, Devi Ahilya University, Indore, India.

Plant material: *Viburnum foetidum* pulverized was a kind gift from AMSAR Private Limited, Indore, India. The plant was identified by Dr. Navin K. Jain at the Department of Botany, Holkar Science College, Indore, India.

Extraction procedure: The dried pulverized bark of *Viburnum foetidum* was macerated in 70% ethanol for 72 h and thereafter, suspension was filtered using Whatman No. 1 filter paper. The filtrate was concentrated at 45°C in order to obtain a solid ethanolic extract. A liquid paste of the ethanolic extract was prepared in distilled water and thereafter partitioned using various solvents of different polarities in the following order: n-hexane, ethyl acetate and n-butanol (one solvent at a time) to obtain the respective fractions[10]. The partitioned fractions were air dried and stored in a refrigerator at 4-8°C temperature prior to use.

Cell lines, media, assay kits and reagents: The RIN-5F pancreatic cells and HepG2 liver cells were purchased from National Centre for Cell Science, Pune-411007, Maharashtra, India. The reagents, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), streptozotocin, insulin assay kit and α-amylase testing kit were purchased from Sigma-Aldrich, USA. Minimum Essential Medium Eagle (MEM), Fetal Bovine Serum (FBS), AccuSure glucometer and blood glucose test strips were procured from Hi-Media, India.

Assay for *in vitro* antidiabetic studies

Maintenance of cell culture: The RIN-5F pancreatic cells and HepG2 liver cells were routinely cultured in MEM supplemented with 10% FBS and 1% streptomycin; and were incubated at 37°C in a CO_2 incubator.

Cell viability assay: The cell lines (RIN-5F pancreatic cells and HepG2 liver cells) were placed separately into 2 different 96-well plates having 6.0×10^3 cells per well in a total 200 µL volume. The cells were left to attach for 48 h and then treated with 100 µL of plant extract at various mass values (0.625, 1.25, 2.5, 5.0 and 10.0 µg/100 µL) to a specific well for 24 h at 37°C. Thereafter, 50 µL MTT (5 mg mL^{-1}) was poured in each well (wells treated with plant extract and wells without treatment) and the contents were put in the dark at 37°C for another 3 h[11]. Thereafter, spent medium was drawn from each well and 200 µL of dimethyl sulfoxide was added to the wells. The optical density was measured at 540 nm using a microplate reader (ECIL Micro Scan MS5608A). The 50% inhibition concentration was determined graphically using Microsoft excel.

RIN-5F cells insulin secretion assay: The RIN-5F pancreatic cells were placed on an average 6.0×10^3 cells per well in a 96 wells plate and incubated for 72 h at 37°C in a CO_2 incubator. Thereafter, the spent medium was aspirated and cells settled in the wells were washed thrice with 200 µL Kreb's Ringer

bicarbonate (KRB) buffer and 10 mM HEPES (pH 7.4) supplemented with 1% bovine serum. Thereafter, the cells were incubated at 37°C for 20 min with 100 µL KRB buffer having 1.1 mM glucose and 100 µL of different mass values of the ethanolic extract (0.625, 1.25 and 2.5/100 µL), 100 µL glibenclamide (1.0 µg/100 µL) and 100 µL phosphate buffered saline (PBS) in place of plant extracts all in different test wells[11]. Thereafter, a portion of the contents was drawn from each well and stored at -20°C before carrying out insulin assay using insulin ELISA kit (Cat No. KAP1251).

HepG2 cells glucose utilization assay: The assay was carried out using the method of Zheng *et al.*[12] with slight modifications. In summary, HepG2 liver cells were seeded at a density of 6.0×10^3 cells per well in a 96 wells plate. After 48 h, spent medium was aspirated and the medium was replaced with 150 µL MEM containing 0.2% BSA and 8 mM glucose. After replacing the medium, a volume of the extracts, metformin and PBS as in insulin assay was added to different wells. After 24 h of incubation, 10 µL of the medium was taken from each well and transferred into another 96 wells plate and then glucose test kit (Cat. No. GLUL 05 100) was used to measure the glucose levels.

Assay for *in vivo* antidiabetic studies: The experiments using Wistar rats were carried out after approval from the Institutional Animal Ethics Committee, Devi Ahilya University, Indore, India (Approval No. 779/CPCSEA/IAEC/2018/010). The approved guidelines were strictly followed.

Animals: Wistar rats of both sexes weighing nearly 200-220 g were provided by the Institutional Animal house of the University. These rats were kept in properly ventilated polypropylene rat cages, fed with broilers mash and were given water *ad libitum.* The rats were allowed to acclimatize with the environment, under natural day light and night conditions for 2 weeks before starting the experiment.

Acute toxicity assay: Three Wistar rats per group (6 groups) were orally (p.o.) acutely administered 10, 100, 1000, 1600, 2900 and 5000 mg extract kg^{-1} body weight (group 1-6, respectively) and were monitored for signs of toxicity/mortality every hour for the first 4 h and daily for 3 days after acute administration of the ethanolic extract[13].

Oral glucose loading: A mass of 10 g glucose kg^{-1} body weight was administered (p.o.) to Wistar rats that were fasted overnight (12 h). After 30 min of administration, blood was drawn from the vein of the tail and the level of blood glucose was checked using glucometer and glucose strips (Accu-Check Active Glucometer, model: GC0088, Mannheim Germany)[14]. Wistar rats with fasting blood glucose level (FBGL) above 7.0 mmol L^{-1} were randomly divided into 8 groups of 5 rats/group, the extracts and glibenclamide were orally administered once. Group 1, normoglycemic Wistar rats (5 mL distilled water only), group 2-4 (100, 200 and 400 mg ethanolic extract kg^{-1} b.wt., respectively), group 5-7 (200 mg n-butanol, ethyl acetate and n-hexane extract fraction kg^{-1} b.wt., respectively), group 8 (5 mg glibenclamide kg^{-1} b.wt.) and group 9, control (10 mL distilled water kg^{-1} b.wt.). The FBGL was taken at 0, 30, 60, 120 and 240 min.

***In vivo* studies in streptozotocin-induced diabetic rats:** Wistar rats were fasted overnight (for 12 h) and diabetes was induced by a single intraperitoneal injection of 60 mg streptozotocin kg^{-1} b.wt. After seventy two hours, blood was drawn from the vein of the tail and the FBGL was measured. Animals with FBGL above 11.1 mmol L^{-1} were selected for the experiment. The streptozotocin-induced diabetic rats were randomly divided into groups as in glucose loaded rats above. The extracts and glibenclamide were administered (p.o.) daily for 21 days and the FBGL was measured[15] on day 0, 4, 7, 10, 14, 18 and 21. On the 21st day, animals were sacrificed and blood samples were collected by cardiac puncture into plain bottles. The serum was collected for insulin[16] and α-amylase assay[17].

Statistical analysis: Data for each group were collected and summarized in a tabular and graph forms for each treatment group. The results were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett *post hoc* multiple comparisons tests at 95% ($p<0.05$) level of significance using Primer (version 3.01). All results were expressed as mean±standard error of mean (SEM).

RESULTS

Effect of *V. foetidum* bark ethanolic extract on the viability of RIN-5F and HepG2 cells: *Viburnum foetidum* bark ethanolic extract caused a significant ($p<0.05$) concentration-dependent decrease in RIN-5F and HepG2 cells viability (Table 1). The ethanolic extract at 0.625, 1.25 and 2.5 µg/100 µL caused no significant change in the percentage of viable RIN-5F and HepG2 cells when compared with the control (untreated cells). However, at higher mass values (5.0 and 10 µg/100 µL), there was a significant ($p<0.05$) decrease in the viable cells (Table 1).

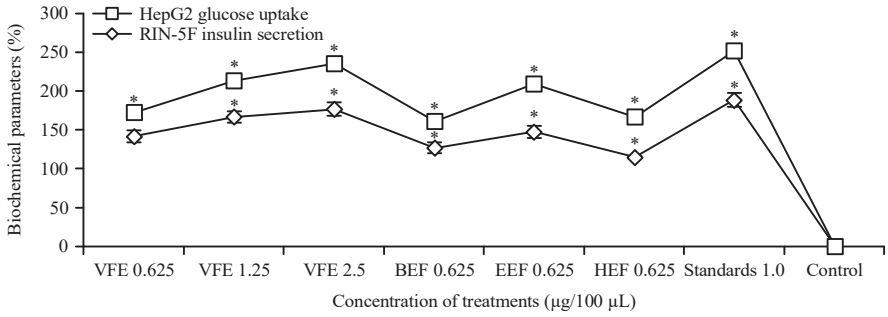

Fig. 1: Effect of *Viburnum foetidum* bark ethanolic extract (VFE) on RIN-5F insulin secretion by RIN-5F and HepG2 glucose uptake

BEF: n-Butanol extract fraction, EEF: Ethyl acetate extract fraction, HEF: n-Hexane extract fraction, standards: Glibenclamide (in insulin assay) and metformin (in glucose utilization assay), values are given as Mean±SEM, n = 3, *p<0.05 compared to the control (untreated cells)

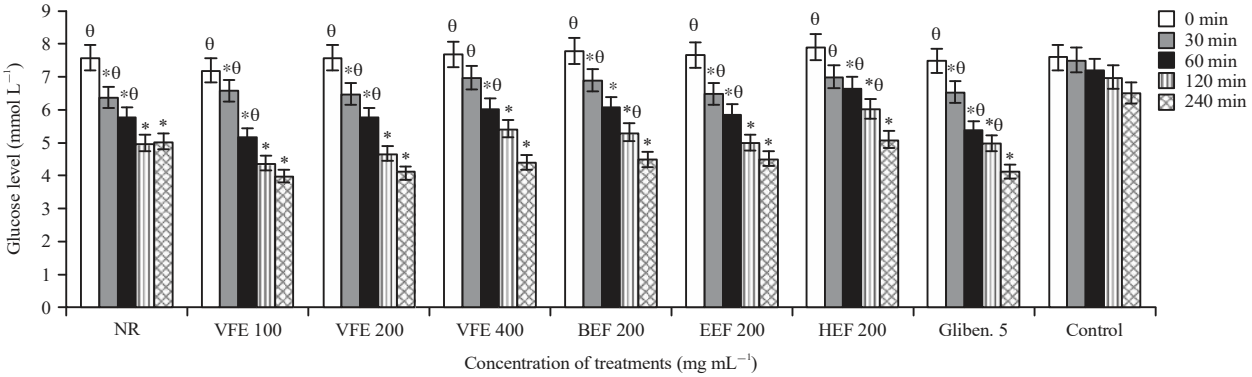

Fig. 2: Effect of *Viburnum foetidum* bark on FBGL (mmol L^{-1}) of glucose loaded Wistar rats

Values are given as Mean±SEM, n = 5, NR: Normoglycemic rats, VFE: *Viburnum foetidum* bark ethanolic extract, BEF: n-butanol extract fraction, EEF: Ethyl acetate extract fraction, HEF: n-hexane extract fraction, Glibencl.: Glibenclamide, *p<0.05 compared to the untreated diabetic rats (control), #p<0.05 compared to group 6 (ethyl acetate fraction), θp<0.05 compared to FBGL at 240 min

Table 1: Effect of *V. foetidum* bark ethanolic extract on the viability of RIN-5F and HepG2 cells

Mass (µg/100 µL)	RIN-5F cell viability (%)	RIN-5F cell death (%)	HepG2 cell viability (%)	HepG2 cell death (%)
VFE 0.625	98.96±1.05	1.04±1.00	98.91±0.71	1.09±1.07
VFE 1.25	97.98±1.07	2.02±0.97	97.99±1.00	2.01±1.14
VFE 2.5	96.80±1.03	3.20±1.16	96.86±0.86	3.14±0.96
VFE 5.0	64.95±1.12*	35.05±1.94	62.05±0.56*	37.95±1.52
VFE 10	43.13±0.92*	56.87±0.81	51.53±1.70*	48.47±0.88
Control	100.00±1.57		100.00±0.92	
IC_{50} of extracts		8.59 µg		9.48 µg

Values are given as Mean±SEM, n = 3, *p<0.05 compared to the control (untreated cells), VFE: *V. foetidum* extract

Effect of *V. foetidum* bark on insulin secretion and glucose uptake: The extracts caused a significant (p<0.05) increase in percentage insulin secretion by RIN-5F and glucose utilization by HepG2 when compared with the control (untreated cells), the ethyl acetate extract fractions caused a higher significant (p<0.05) increase than other extract fractions (Fig. 1).

Median lethal dose (LD_{50}) of *Viburnum foetidum* bark ethanolic extract: The ethanolic extract caused no mortality in Wistar rats after acute administration of the extract orally and the LD_{50} of the extract was ≥5000 mg kg^{-1} b.wt.

Effect of *V. foetidum* bark on fasting blood glucose levels: The extracts and glibenclamide caused a significant reduction in the fasting blood glucose level from 30 min when compared with the control (untreated Wistar rats) and 240 min in glucose loaded rats (Fig. 2). In streptozotocin-induced diabetic rats, the extracts caused a significant reduction in fasting blood glucose level when compared with the control and on the 21st day when compared with day 1, 4, 7, 10, 14 and 18 (Fig. 3). The ethyl acetate extract fraction at 200 mg kg^{-1} exerted a more significant reduction when compared with other extract fractions on day 7 and 10 (Fig. 3).

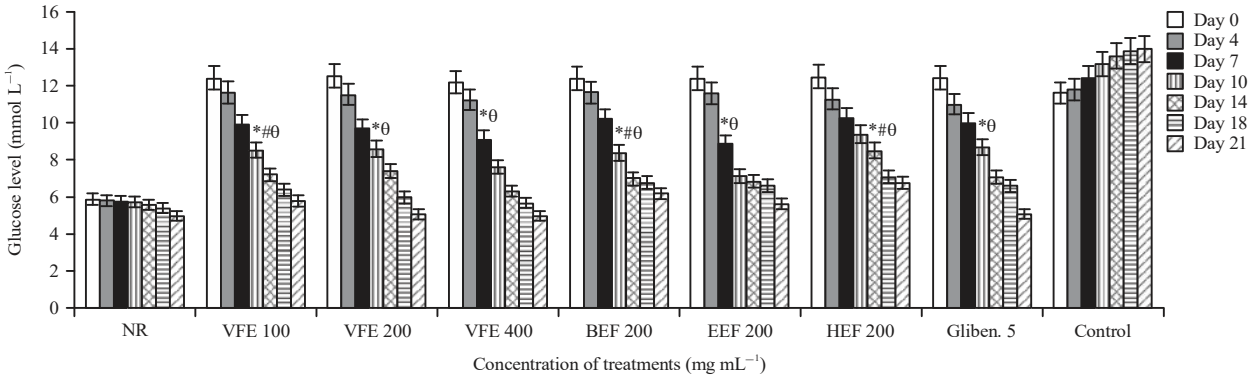

Fig. 3: Effect of *Viburnum foetidum* bark on FBGL (mmol L^{-1}) of streptozotocin-induced diabetic Wistar rats

Values are given as Mean±SEM, n = 5, NR: Normoglycemic rats, VFE: *Viburnum foetidum* bark ethanolic extract, BEF: n-butanol extract fraction, EEF: Ethyl acetate extract fraction, HEF: n-hexane extract fraction, Gliben.: Glibenclamide, *p<0.05 compared to the untreated diabetic rats (control), #p<0.05 compared to group 6 (ethyl acetate fraction), θp<0.05 compared to FBGL in day 21

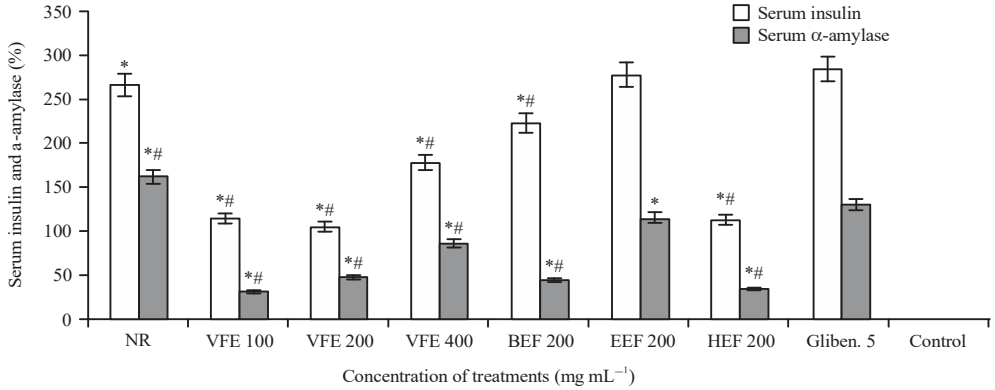

Fig. 4: Effect of *Viburnum foetidum* bark extracts on serum insulin and serum α-amylase levels (U L^{-1})

Values are given as Mean±SEM, n = 5, *p<0.05 compared to untreated diabetic rats (control), #p<0.05 compared to group 6 (ethyl acetate fraction). NR: Normoglycemic rats, VFE: *Viburnum foetidum* bark ethanolic extract, BEF: n-butanol extract fraction, EEF: Ethylacetate extract fraction, HEF: n-hexane extract fraction, Gliben.: Glibenclamide

Effect of *V. foetidum* extracts on serum insulin and serum α-amylase levels: The plant extracts and glibenclamide caused a significant increase in serum insulin and serum α-amylase level of Wistar rats when compared with the untreated diabetic rats (control) (Fig. 4). The ethyl acetate extract fraction at exerted a more significant increase when compared with the ethanolic extract and other extract fractions (Fig. 4).

DISCUSSION

In the present study, the antidiabetic mechanisms of *Viburnum foetidum* bark ethanolic extract and ethanolic extract fractions (n-butanol, ethyl acetate and n-hexane) were investigated using both *in vitro* and *in vivo* models. The results of the *in vitro* study showed that the extracts enhanced RIN-5F insulin secretion and HepG2 glucose uptake. Whereas, in the *in vitro* study, the plant extracts caused reduction in FBGL (in both glucose loaded and streptozotocin-induced diabetic rats), increased serum insulin and serum α-amylase levels in streptozotocin-induced diabetic rats. The cells were viable at lower concentration and there was no mortality up to 5000 mg kg^{-1} b.wt., ethanolic extract (p.o.).

The hormone, insulin produced by the islets of Langerhans beta cells found in the pancreas functions in the uptake of glucose by the body cells which include fat, muscle and liver cells[18]. In type 2 diabetes, the pancreas is unable to produce enough insulin or the insulin is not sensitive to cells[19]. In this study, the bark of *Viburnum foetidum* increased HepG2 glucose uptake, enhanced RIN-5F insulin secretion and increased serum insulin levels in streptozotocin-induced diabetic rats. These results are similar to previous antidiabetic studies results carried out on plant extracts[20-22].

Oral glucose loading model for induction of diabetes in experimental animals also known as glucose tolerance testing (GTT) indicated increase in the blood glucose level without damaging the pancreas[23]. Whereas, streptozotocin exerts its diabetogenic action in experimental animals by selectively destroying the insulin secreting beta cells of the pancreas[23,24]. In this study, *Viburnum foetidum* bark extracts reduced the FBGL from 30 min in glucose loaded rats and from the 7th day onwards in streptozotocin-induced diabetic Wistar rats. The ethyl acetate extract fractions caused a more reduction than the other extract fractions.

Apart from the salivary glands, the pancreas also secretes the enzyme α-amylase which catalyzes digestion of starchy carbohydrate to glucose[25]. In diabetic condition, the islets of the pancreas are damaged and this affects the synthesis and release of α-amylase from the pancreas[26,27]. Recent clinical studies also reported that low serum α-amylase was observed when there is high blood glucose level in type 2 diabetes and this signifies a defect in cells of the pancreas[26,28]. In this study, there was an increase in serum α-amylase levels in diabetic treated animals and this further revealed the healing effect of the plant extract on the pancreatic cells in diabetic condition.

The enzyme α-amylase which catalyzes digestion of starchy carbohydrate to glucose is secreted by the β-cells of the pancreas[25]. The β-cells of the pancreas are damaged in diabetic condition and this affects the synthesis and release of α-amylase[26,27]. Recent clinical studies reported that in type 2 diabetes condition there was low serum α-amylase level due to a damage in the pancreatic cells[26,28]. *Viburnum foetidum* bark caused an increase in serum α-amylase levels in diabetic treated animals in this study.

The standards (known antidiabetic drugs) used in this study for the investigation of the mechanism of action of *Viburnum foetidum* bark have different antidiabetic mechanisms of action which include; enhancement of insulin secretion by the pancreas (glibenclamide) and reduction of glucose production by the liver (metformin)[29]. The results from this study showed that the possible antidiabetic mechanism of *Viburnum foetidum* bark is by increasing insulin secretion by the pancreas and reducing glucose production by the liver.

CONCLUSION

To conclude, the results of this study showed that *Viburnum foetidum* bark has potent antidiabetic activity and the possible mechanism of action is by enhancing secretion of insulin, increasing glucose uptake by hepatic cells and enhancing the functionality of the pancreas thereby increasing serum insulin and serum α-amylase level. The results also showed that ethyl acetate extract fraction has a more antidiabetic activity than the other extract fractions.

SIGNIFICANCE STATEMENT

This study discovered that *Viburnum foetidum* bark exerted antidiabetic effect by enhancing secretion of pancreatic insulin and α-amylase. This study will help researchers to uncover the critical areas in the use of the plant for treatment of diabetes that many researchers were not able to explore. Thus a new theory in the treatment of diabetes will be uncovered.

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