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Antidiabetic activity of aqueous stem bark extract of *Annickia polycarpa* in alloxan-induced diabetic mice

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ABSTRACT

Background and aim: There is a growing need to develop new drugs for type II diabetes mellitus (DM) from plant sources due to the high cost and adverse side effects of current drug therapies. To this end, the antidiabetic activity of aqueous stem-bark extract of *A. polycarpa* (APE) in alloxan-induced diabetic ICR mice was investigated.
Experimental procedure: The effect of APE (20, 100 and 500 mg/kg), glibenclamide and metformin as positive controls, were determined over 4 weeks on fasting blood glucose (FBG). An oral glucose tolerance test (OGTT) was also conducted. The effects of these treatments on the morphology of the pancreas were assessed. In addition, phytochemical constituents and antioxidant properties of APE were determined.
Results and conclusion: APE, like glibenclamide and metformin, showed significant hypoglycaemic effect. The OGTT supported the hypoglycaemic effect. The destroyed pancreatic beta-cells in diabetic control mice were restored to normal by APE or drug treatment. APE showed antioxidant activity by scavenging DPPH free radicals; this may be due to the presence of phenolic compounds, particularly flavonoids. Thus, APE may act by restoring pancreatic beta-cell integrity through mopping of reactive oxygen species (ROS) associated with the diabetic state, and thereby improving pancreatic function and consequently, the lowering of FBG levels. These findings provide ample evidence to validate the traditional use of *A. polycarpa* in the management of DM.
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1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterised by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. There are generally two types of DM; type I and type II. Type I, also called insulin-dependent DM in which there is the absolute lack of insulin and type II DM also called non-insulin dependent DM due to impaired secretion and action of insulin.[1] Dm is a leading cause of morbidity and mortality worldwide, with an estimated worldwide prevalence among adults in 2011 to be 346 million (6.1%) according to WHO.[2] This number is predicted to rise to around 439 million (7.7%) by 2030.[3] WHO projects that deaths resulting from diabetes will increase by two thirds between 2008 and 2030.[2]

In urban Ghana, type II DM affects predominantly obese patients of rather low socioeconomic status and is frequently accompanied by hypertension and hyperlipidaemia.[4] Some 23 % of adults have been known to be overweight, and this has been linked to age, gender, urbanisation, income and educational status.[5,6] Once diagnosed, DM is managed using various therapeutically effective drugs like insulin for type I DM, and oral hypoglycaemic agents (glibenclamide and metformin) for type II DM. However, these drugs are generally not cost-effective and also possess adverse side-effects. Between 60 and 95% of the Africans are said to depend on

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List of abbreviations

APE	Annickia polycarpa extract
AUC	Area under the curve
BHT	Butylated hydroxytoluene
CPMR	Center for Plant Medicine Research
DM	Diabetes mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl
FBG	Fasting blood glucose
GA	Gallic acid
GAFCO	Ghana Agro Food Company Limited
GLIB	Glibenclamide
ICR	Institute of cancer Research
IDDM	Insulin-dependent diabetes mellitus
MET	Metformin
OGTT	Oral glucose tolerance test
QE	Quercetin
ROS	Reactive oxygen species
SEM	Standard error of the mean
WHO	World Health Organization

traditional medicine for their primary health care needs.[7] Thus, medicinal plants have an ever-increasing role to play in the treatment or management of lifelong diseases like DM, especially in developing countries where resources are meagre, and the plants are readily available.[8]

Over the past few decades, there has been a resurgence of interest in the investigation of plant materials as sources of potential medicinal substance[9] and these plant-based medicines are referred to as herbal medicines. In Ghana and elsewhere, *Indigofera arrecta*, *Bridelia ferruginea*, unripe fruit of *Musa paradisiaca*, *Momordica charantia*, and *Ocimum canum* have all been reported to possess antidiabetic properties.[10–14] Most of these plants contain phytochemicals that possess antioxidant properties, which could help overcome oxidative stress associated with the diabetic state.[15,16]

The bark and leaves of *A. polycarpa* contain many biologically active alkaloids such as berberine and protoberberines.[17,18] These compounds are probably responsible for the remarkable antitrypanosomal activity.[19] Berberines and protoberberines have been linked to lowering hyperglycemia, reducing insulin resistance, stimulating pancreatic beta-cell regeneration, and decreasing lipid peroxidation in mouse type II diabetes model.[20] There is anecdotal and traditional evidence of the use of APE for the management of DM. We aimed to validate this claim by investigating the antidiabetic and antioxidant properties of the stem bark of the plant.

2. Materials and methods

2.1. Materials

2.1.1. Reagents and chemicals

Glucose assay kit was obtained from ELI Tech (Puteaux, France). Glibenclamide and metformin were obtained from BLISS GVS PHARMA Limited (Mumbai, India). Diphenylpicrylhydrazyl was purchased from Sigma Chemical Company (St. Louis, MO, USA). Urine test strips (UroColor™10) were supplied by Standard Diagnostics Inc. (Kyonggi-do, S. Korea). All other chemicals and reagents used were obtained from FLUKA (Zofingen, Switzerland).

2.1.2. Plant material

The stem bark of *A. polycarpa* was collected from Begoro, Eastern Region, Ghana (6°23′N 0°23′W) and authenticated by Mr. H.

Blagogee, of the Plant Production Department (PDD), Centre for Plant Medicine Research (CPMR), Mampong-Akuapem in the Eastern Region of Ghana, and deposited with the herbarium of the PDD with voucher specimen number 01/2015. The plant is cultivated on a large scale by the PDD of the CPMR for sustainable purposes.

2.1.3. Experimental animals

Male ICR mice (20–30 g) were obtained from the Animal Experimentation Unit, CPMR. The animals were housed in stainless steel metal cages, sized 20.3 cm width × 28.7 cm length × 17.3 cm height, with wood shavings as bedding and fed on powdered feed obtained from GAFCO (Tema, Ghana) and allowed free access to sterilised distilled water. The experimental animals were in an environment of $22 \pm 2\ °C$ temperature and $70 \pm 4\%$ humidity under alternating 12 h period each of light and darkness. They were put in seven groups of 5 animals each. All animals were handled following the Guide for the Care and Use of Laboratory Animals. Ethical clearance for the study was obtained from the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana, Korle-Bu, Accra (Protocol Identification Number: CHS–Et/M.4 – P 3.10/2015–2016).

2.2. Methods

2.2.1. Preparation of plant material

The fresh stem bark of *A. polycarpa* (AP) was air-dried at 25 °C for 1 week and then milled into powdered form in a laboratory mill. The powdered stem bark was weighed and boiled in water (20 % w/v) for 30 min, allowed to cool and filtered through cotton wool. The filtrate was concentrated by a rotary evaporator and lyophilised into powder by a freeze dryer (EYELA, Tokyo Rikakikai Co. Ltd, Japan). The aqueous freeze-dried extract of AP, subsequently referred to as APE, was weighed and stored in a desiccator at 4–8 °C. The % yield of extract from plant material was calculated.

2.2.2. Qualitative phytochemical screening

Qualitative phytochemical tests were performed on APE to determine the presence of saponins, cyanogenic glycosides, alkaloids, flavonoids, reducing sugars, phenolic substances, polyuronides, anthracenosides, triterpenes and phytosterols according to standard protocols.[21] For each test, a small amount (1–2 g) of APE was reconstituted in distilled water/another solvent before use.

2.2.3. Quantitative phytochemical analysis

2.2.3.1. Total phenolic content.
The total phenolic content of the APE was determined by the Folin-Ciocalteau method.[22] A volume of 100 μl of 5% Folin-Ciocalteau reagent was added to 20 μl of 0–5 mg/ml of the APE followed by 80 μl of 7.5% Na_2CO_3 in a well plate and mixed. The mixture was then incubated at room temperature in the dark for 1 h and the absorbance measured at 765 nm using the Infinite M200Pro microtiter plate reader (Tecan, Austria). Gallic acid at a concentration range of 0–5 mg/ml was used to plot the standard calibration curve from which the concentration of phenol in each APE concentration was estimated. The total phenolic content of the extract was expressed in mg of Gallic acid equivalents (GAE)/g of APE for triplicate determinations.

2.2.3.2. Determination of total flavonoids.
The total flavonoid content of the APE was determined by the slightly modified aluminium chloride colourimetric method.[23] Various concentrations of APE in distilled water were prepared by serial dilution; (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, 0.0391 mg/ml). Briefly, a volume (100 μl) of 2 % $AlCl_3$ was added to 100 μl of the APE (0.0391–5 mg/ml), followed by incubation in the dark for 10 minutes at room temperature. The absorbance of the solution was measured at a

wavelength of 415 nm using the Infinite microtiter M200Pro plate reader. Quercetin (0−5 mg/ml) was used as a standard while distilled water was used as a blank. A calibration curve for quercetin was plotted from which the unknown concentration of flavonoids in each APE concentration was estimated. The total flavonoid content of APE was expressed in mg of quercetin equivalents (QE)/g of APE for triplicate determinations.

2.2.4. Induction of diabetic state in mice

The diabetic state was induced in normoglycaemic ICR mice by a single intraperitoneal injection of alloxan monohydrate (150 mg/kg).[24] The alloxan was first weighed individually for each animal according to the body weight and then dissolved in 0.5 ml saline (0.9 % NaCl) just before administration to the animal. Two days after alloxan administration, the fasting blood glucose (FBG) of mice was determined, and those with FBG $\geq$ 7.8 mM were classified as diabetic and, therefore, included in the study. Treatment with APE commenced 48 h after establishing the diabetic state of animals.

2.2.5. Treatment of animals

The alloxan-induced diabetic mice were divided into six (6) treatment groups of five (5) animals each. The first three groups were treated daily with APE of increasing dosage (20, 100 and 500 mg/kg). Members of the 4th group were treated daily with the maximum therapeutic dose of glibenclamide (2.5 mg/kg) whereas the 5th group received daily the maximum therapeutic dose of metformin (50 mg/kg). The 6th group received an equivalent volume of water (0.5 ml) and served as the diabetic control group. Five normoglycaemic littermates were put in a 7th group, which received an equal volume of water (0.5 ml) and represented the non-diabetic control. The animals were administered with/without APE or drug for four weeks by oral gavage. APE or standard drugs were reconstituted in sterilised distilled water before administration to the animals. The body weights of the animals in each group were determined weekly.

2.2.5.1. Blood sampling.
At baseline, and the end of weeks 1, 2, 3 and 4, blood samples were collected by tail bleeding of the animals after an overnight fast (8 h) into serum separator tubes and centrifuged at 4500×g for 5 min to obtain serum The serum was transferred into fresh tubes and stored at −80 °C until use.

2.2.5.2. Serum glucose determination.
Plasma glucose concentration was determined by the glucose oxidase method by following the manufacturer's protocol in assay kits (ELI Tech, Puteaux, France).), and measuring absorbance by the Biosystems A25 Chemistry Analyzer (Biosystems S.A, Barcelona, Spain).

2.2.5.3. Oral glucose tolerance test.
The oral glucose tolerance test was conducted on the same animals at the termination of treatments. The animals of all treatment groups were fasted for 8 h, and the FBG determined at baseline. Each animal was given an oral glucose load (2 g/kg), by administering about 0.5 ml/animal (depending on body weight) of glucose solution. Blood samples were collected by tail bleeding as previously, at 30, 60, 90, 120 and 150 min post-glucose load. FBG levels were measured using the glucose oxidase method.

2.2.5.4. Histology of pancreas.
Animals were killed at the end of the study by cervical dislocation. The pancreas was removed, washed with cold saline and preserved in 10% buffered formalin. Blocks from tissues were routinely processed and embedded in paraffin. Thin sections were cut using microtome and stained with hematoxylin and eosin for histopathological evaluation by light microscopy.[25]

2.2.6. Antioxidant activity

2.2.6.1. DPPH radical scavenging assay.
The assay was performed according to a previously established method.[26] Briefly, a fresh solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in 80 % methanol (0.5 mM) was prepared. Various concentrations (0.625, 1.25, 2.5 mg/ml) of APE were prepared by serial dilution of an initial stock solution of 10 mg/ml in methanol. 100 μl of DPPH solution was added to 100 μl of each extract in a 96 μL well plate. The plate was shaken to uniformly mix the solution and kept in the dark for 30 min. The absorbance was read at 517 nm using Infinite M200Pro microtiter plate reader (Tecan, Austria). Butylated hydroxytoluene (BHT) at concentrations as the extract in methanol was used as positive control and 80 % methanol as blank.

The % DPPH scavenging activity of APE was calculated using the formula below:

$$\text{\% DPPH scavenging effects} = (Ac - At) \times 100/Ac$$

Where: Ac = Absorbance of control, As = Absorbance of the standard/extract.

A graph of percentage DPPH scavenging activity was plotted against the various concentrations of APE.

2.2.6.2. Ferric ion reducing ability.
The ferric ion reducing ability of APE was determined with Gallic acid, BHT and quercetin as standards.[27] 0.5 ml of increasing concentration (0−5 mg/ml) of APE was added to 0.5 ml of distilled water and mixed with 1.25 ml of sodium phosphate buffer (0.2 M, pH = 6.6), followed by 1.25 ml of 1 % potassium ferric cyanide. After incubation at 50°C for 20 min, 1.25 ml of 10 % trichloroacetic acid were added to each sample and centrifuged at 3000×g for 10 min. 100 μl of distilled water and 100 μl of 0.1 % FeCl₃ were added to 100 μl of supernatant APE in a plate well. The absorbance of the resulting solutions was read at 700 nm by Infinite M200Pro microtiter plate reader (Tecan, Austria). A blank was prepared by adding 0.5 ml of distilled water to 1.25 ml of sodium phosphate buffer and 1.25 ml of potassium ferricyanide. A graph of absorbance against the concentrations was plotted for APE and the standards.

3. Results

3.1. Qualitative phytochemical analysis

The preliminary phytochemical screening of APE (5% yield = 50 g extract/kg plant material) showed the presence of saponins, reducing sugars, phenolic compounds, alkaloids and flavonoids.

3.2. Quantitative phytochemical analysis

The phenolic and flavonoid contents of APE increased with increasing concentration, reaching a plateau at 2 mg/ml (Fig. 1). The total phenolic and flavonoid contents of APE were 62.2 ± 4.1 mg GAE/g extract and 662.8 ± 24.2 mg QE/g extract, respectively.

3.3. Antioxidant activity

The DPPH free radical scavenging activity of APE and standard antioxidant BHT indicated that APE at the concentrations used, showed a concentration dependency while BHT did not (Fig. 2). The free radical scavenging activity of BHT at the highest concentration of 2.5 mg/ml was 75.04 % whereas that of APE at the same concentration was 45.07 %. In the *in vitro* Fe^{3+} ion reducing power studies, there was a concentration-dependent increase in ferric reducing ability in respect of Gallic acid and quercetin, reaching a
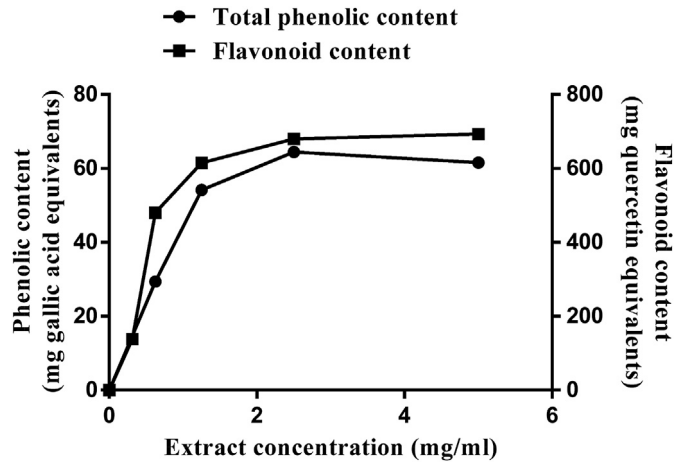
Fig. 1. Total phenolic and flavonoid contents of increasing concentration of APE. Values are means ± S.E.M. of n = 3. APE = *A. polycarpa* aqueous stem bark extract.
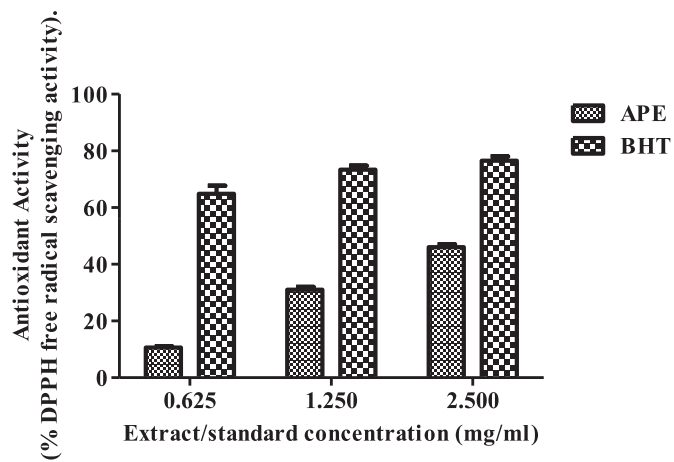

Fig. 3. The ferric ion reducing activity of varying concentrations of quercetin, Gallic acid, butylated hydroxytoluene (BHT) and *A. polycarpa* aqueous stem bark extract (APE). Results are means ± S.E.M of n = 3.

Fig. 2. DPPH free radical scavenging activity of varying concentrations of APE and the standard BHT. Results are means ± S.E.M of n = 3. APE = *A. polycarpa* aqueous stem bark extract; BHT = butylated hydroxytoluene.

plateau after the concentration of 1.25 mg/ml. BHT and APE at varying concentrations did not show much ferric ion reducing capability (Fig. 3).

3.4. Fasting blood glucose (FBG) levels

The FBG level in diabetic mice was two-fold that of the normoglycaemic control at baseline (Fig. 4a), and these levels were maintained throughout the study. FBG levels in diabetic animals were significantly reduced (p < 0.05) by APE over time, reaching near normoglycaemic control value by week 4 as seen with the standard drugs metformin and glibenclamide. This is depicted in the area under the curve where similar degrees of increase in total FBG were observed in diabetic mice relative to the normoglycaemic control and similar degrees of reduction on treatment with APE or standard drugs relative to diabetic controls (Fig. 4b).

3.5. Oral glucose tolerance test

At baseline, the FBG level of diabetic controls was 3-fold that of normoglycaemic controls while those of other treatment groups were in between these two control groups. Administration of an
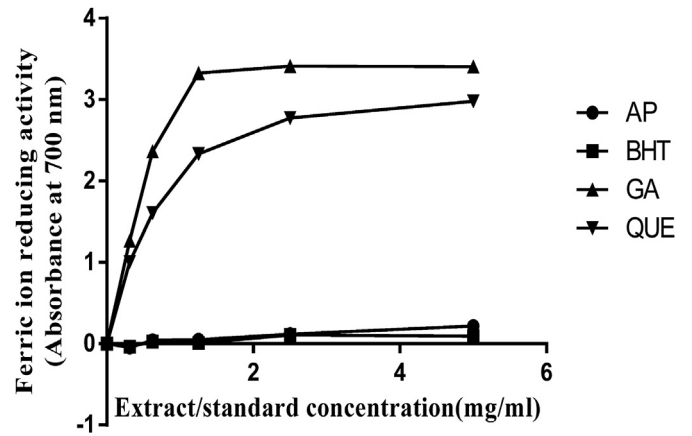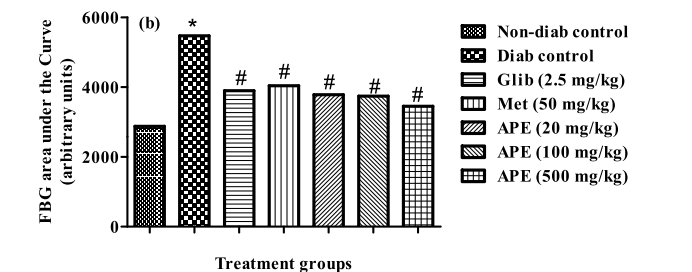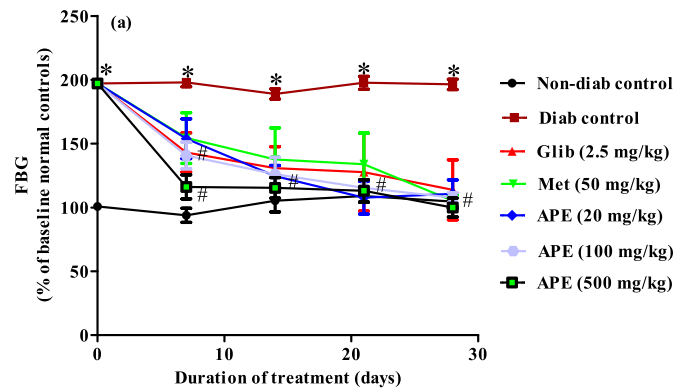

Fig. 4. Effect of APE and standard drugs on FBG levels; (a): line graph and (b): area under the curve (AUC). Values are percentages of baseline normoglycaemic control FBG (4.3 ± 0.1 mmol/L). Results are means ± S.E.M of n = 5. Glib = glibenclamide; Met = metformin; APE = *A. polycarpa* aqueous stem bark extract; FBG = fasting blood glucose. *Value significantly different from normoglycemic control (p < 0.05). #Value significantly different from diabetic control (p < 0.05).

oral glucose load to diabetic control mice caused about a 3-fold increase in baseline blood glucose level within 30 min with a gradual decline to above baseline level at 150 min. The blood glucose of normoglycaemic controls increased 2.5-fold in 30 min and reduced to baseline levels by 60 min and remained at that level up to 150 min. The blood glucose levels of diabetic mice treated with the standard drugs and APE responded similarly as the normoglycaemic controls after the glucose load (Fig. 5a). The general effects of treatments on the management of the glucose load

throughout the study are depicted in the AUC (Fig. 5b), which showed that in animals treated with APE or standard drugs the marked elevation in blood glucose level in diabetic mice after the glucose load was significantly reduced (see Fig. 5).

3.6. Changes in mean body weights

There was a gradual increase in mean body weights across all animal treatment groups throughout the study. The changes in body weights of APE or drug-treated diabetic animals appeared to have reached near normoglycaemic control value but significantly higher than the diabetic control value at the termination of treatments (Fig. 6).

3.7. Histopathology of pancreas

Fig. 7 shows the micrographs of the pancreas at the termination of treatment of diabetic mice with APE and standard drugs. The diabetic control group showed the destruction of the beta cells,

while the normoglycemic control group showed intact pancreatic beta cells. Treatment with metformin, glibenclamide and APE facilitated the restoration of the morphology of the beta cells of the pancreas in the diabetic mice.

4. Discussion

The study was conducted to evaluate the antidiabetic effect of aqueous *Annickia polycarpa* stem bark extract (APE) in alloxan-induced diabetic mice with the view to validating its traditional use in the management of diabetes mellitus (DM) in humans. DM is a medical condition characterised by chronic hyperglycemia caused by a relative or absolute deficiency of insulin or resistance to the action of insulin at the cellular level.[28] Alloxan-induced hyperglycaemia is considered as a suitable experimental model to study the hypoglycaemic effect of antidiabetic agents in type II DM. In alloxan-induced DM, there is the destruction of the beta cells by free radicals produced by alloxan, which leads to impaired pancreatic function and reduced secretion of insulin and
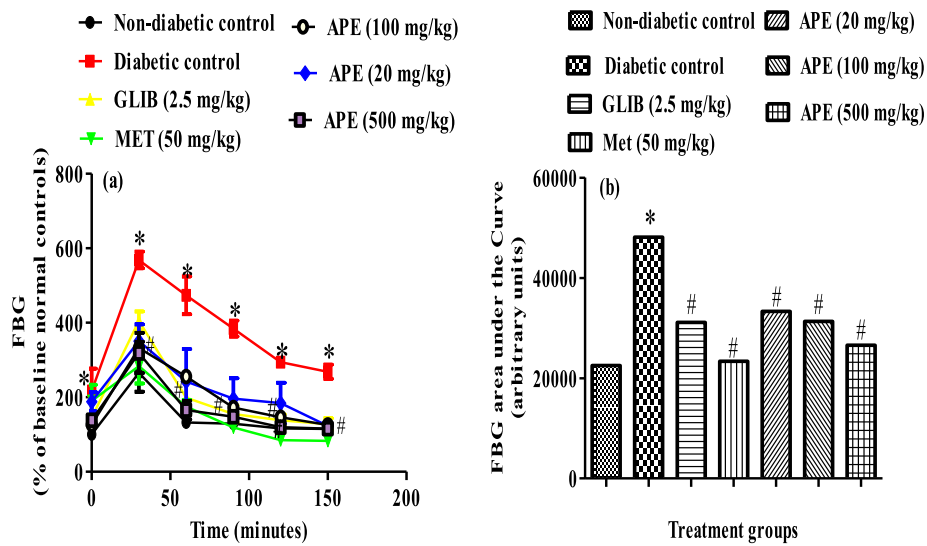
Fig. 5. Effect of APE and standard drugs on FBG levels after an oral glucose load; (a): line graph; (b): area under the curve (AUC). Values are percentages of baseline normal control fasting blood glucose of (4.5 ± 0.2) mmol/L. Glib = glibenclamide; Met = metformin; APE = *A. polycarpa* aqueous stem bark extract; FBG = fasting blood glucose. Results are mean $\pm$ S.E.M of n = 5. *Value significantly ($p < 0.05$) different from normoglycaemic control. #Value significantly ($p < 0.05$) different from diabetic control.
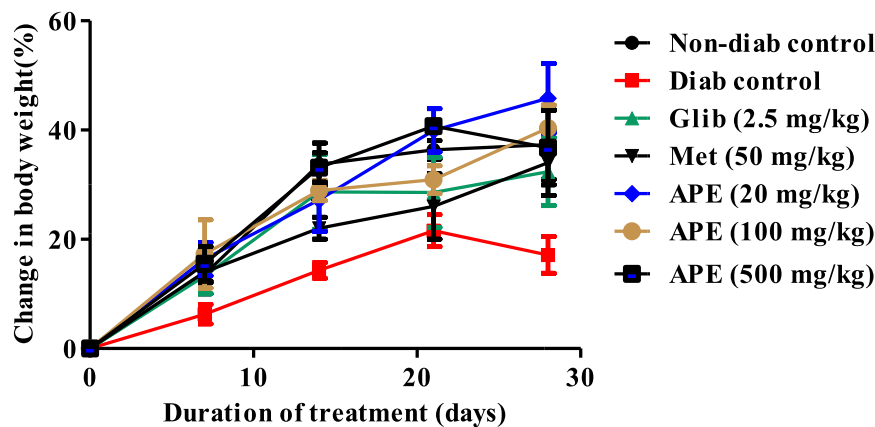
Fig. 6. Weekly percentage changes in body weight of Non-diabetic/diabetic mice over the duration of treatment with APE and standard drugs. Values were expressed as the percentage change in baseline normoglycaemic control group of weight 27.6 ± 0.6 g. Glib = glibenclamide; Met = metformin; APE = *A. polycarpa* aqueous stem bark extract. Results are means $\pm$ S.E.M of n = 5.
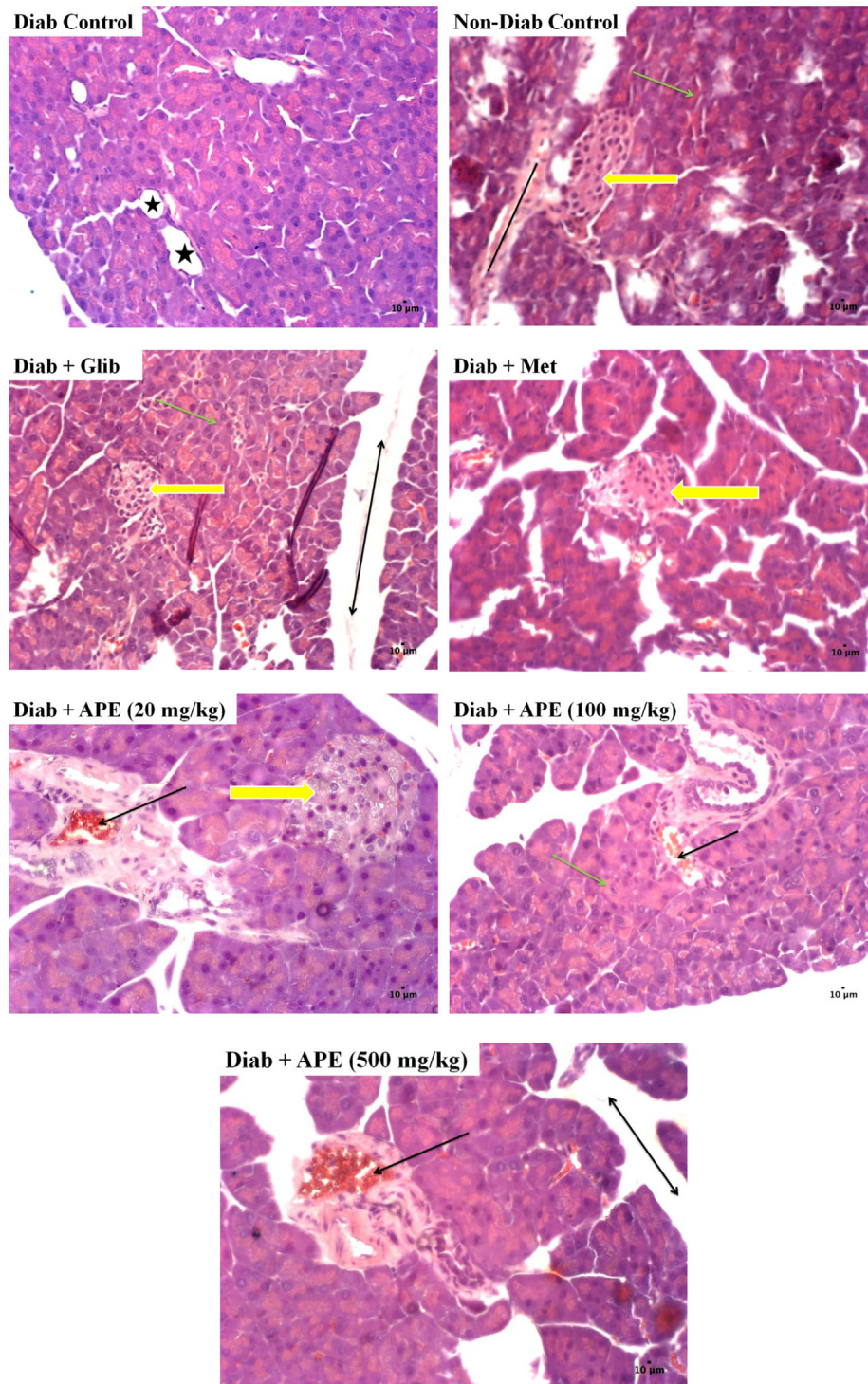
Fig. 7. Histological appearance of mice pancreas at termination of treatments for Diab Control: diabetic control animals, Non-Diab control: normoglycaemic control animals, Diab + Glib: diabetic animals treated with glibenclamide, Diab + Met: diabetic animals treated with metformin, Diab + APE (20 mg/kg): diabetic animals treated with treated with APE (20 mg/kg), Diab + APE (100 mg/kg): diabetic animals treated with APE (100 mg/kg) and Diab + APE (500 mg/kg): diabetic animals treated APE (500 mg/kg) showing destruction of pancreatic beta cells and disintegrating acinar cells in diabetic mice and the restoration of the pancreatic beta cells in various degrees in other treatment groups compared to normoglycaemic controls. The thick yellow arrow represents islets of Langerhans; Star represent destroyed islets, double end arrow represents interlobular duct, a green arrow indicates pancreatic acinar cells and the black arrow represents infiltration of neutrophils. APE = *A. polycarpa* aqueous stem bark extract. Magnification: ×200. Scale bar is 10 μm.

consequently hyperglycaemia.[29]

In this study, male ICR mice were made diabetic by a single intraperitoneal injection with alloxan (150 mg/kg body weight) followed by treatment for 4 weeks with APE and the standard drugs, glibenclamide and metformin. Treatment of alloxan-induced diabetic mice with the extract significantly reduced the glucose levels throughout study, reaching near normoglycaemic levels by the termination of treatments (Fig. 4), suggesting that the extract

possesses hypoglycaemic activity. The hypoglycaemic activity of the extract was comparable to that of glibenclamide and metformin at the doses used. Metformin acts by increasing glucose absorption into peripheral tissues and glibenclamide acts by increasing insulin production. The hypoglycaemic effect displayed by APE may be due to improvement in pancreatic function as a result of the restoration of the morphology of beta cells of the pancreas that had being destroyed in the diabetic mice (Fig. 7). This recovery was not dose-dependent with the APE dosage of 20 mg/kg showing a much better recovery of the pancreatic beta -cells. The presence of alkaloids and phenolic compounds, especially flavonoids in the extract may be responsible for the observed hypoglycaemic effect of the extract.[30,31] The glucose tolerance test further revealed that APE significantly improved the ability of diabetic mice to handle an oral glucose load in a fashion similar to that of glibenclamide and metformin (Fig. 5).

DM leads to the generation of excess quantities of free radicals due to hyperglycaemia and hyperinsulinaemia[16,32] and this result in oxidative stress. Oxidative stress plays a role in the progression of DM and its complications. Diabetic patients have insufficient anti-oxidant defenses,[15,33] and for that reason, any treatment regimen for DM should of necessity have antioxidant activity to reduce diabetic complications. Antioxidants are compounds that show reducing activity. They keep the components of cells and bio-molecules from oxidation by donating an electron/hydrogen atom to free radicals/reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxyl radicals. Studies have proven the beneficial role antioxidants play in DM.[15,34] Plant phenolics are essential because they possess hydroxyl groups that confer scavenging ability. APE was shown to contain considerable quantities of phenolic compounds, particularly flavonoids (Fig. 1).

The antioxidant effect of flavonoids is the possible reason why they prevent glycation.[35] Studies have shown that flavonoids are highly effective scavengers of most oxidising molecules which include singlet oxygen and various free radicals[36] implicated in several diseases. The antioxidant activity of the extract may involve the scavenging of free radicals as seen with DPPH (Fig. 3), thereby protecting cells from the damaging effect of the free radicals and successive complications. The antioxidant activity of a substance is also measured by its reducing ability by donating a hydrogen atom which helps disrupt free radical chains, thereby breaking them.[37] The extract appeared to have little to no reducing ability (Fig. 4) confirming that the antioxidant activity of the extract is as a result of the scavenging of free radicals.

5. Conclusions

In conclusion, the aqueous stem bark extract of *A. polycarpa* (APE) possesses significant antidiabetic activity due to its hypo-glycaemic and antioxidant effects. This antidiabetic effect may be due to its ability to cause the regeneration of beta cells of the pancreas and thus the improvement in pancreatic function. The extract by its possession of significant antioxidant activity may scavenge ROS and thus help in overcoming some of the advanced complications of DM. The presence of alkaloids and phenolics, mainly flavonoids may be responsible for the hypoglycaemic activity of APE and the latter its antioxidant effects. These findings support the traditional and anecdotal use of the plant in the management of DM.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2020.02.001.

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