Preliminary study: Pharmacotherapeutic effect of the aqueous stem cell extract of Swiss apple {Malus domestica (Borkh)} of the Uttwiler Spätlauber specie in rats

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Abstract

Background: Diabetes impacts negatively many aspects of global development including economic sustainability and human development. The burden of diabetes with its increasing prevalence causes a harsh financial decline hence, the quest for a permanent solution. Plant stem cell therapy has the potential to drastically change the narrative of scientific research from the perspective of being reactive to preventive and restorative. Objective: This study sought to determine the pharmacotherapeutic effect of the aqueous stem cell extract of Malus domestica (MD) in rats Method: Antidiabetic effect of the aqueous extract of MD (50,100, 200, and 400 mg/kg) was investigated in normoglycemic and oral glucose-induced hyperglycemic rats that were fasted overnight. Furthermore, acute oral toxicity studies were conducted using the limit dose test of the Up-and-Down Procedure according to OECD/OCDE test guidelines on acute toxicity. In addition, ∝-amylase and ∝-glucosidase enzyme inhibitory assays, phytochemical analysis, and antioxidant activity were assessed. Results: The phytochemical constituents exhibited flavonoids, phenols, phlobatannins, reducing sugars, carbohydrates, steroids, and cardiac glycosides. No mortality or signs of toxicity after oral administration with a single dose of 5 g/kg of the MD aqueous extract was recorded. Inhibition of ∝-amylase and ∝-glucosidase, positive antioxidant scavenging activity, and a significant ($p < 0.0001$) reduction in blood glucose level in the hyperglycemic-induced rats but not the normoglycemic were discovered. Pearson correlation showed an association between 50 and 100 mg/kg of MD and glibenclamide. Conclusion: There could be a strong correlation between MD as a potential antidiabetic drug due to the antioxidant properties it possesses.

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Background: Diabetes impacts negatively many aspects of global development including economic sustainability and human development. The burden of diabetes with its increasing prevalence causes a harsh financial decline hence, the quest for a permanent solution. Plant stem cell therapy has the potential to drastically change the narrative of scientific research from the perspective of being reactive to preventive and restorative.This study sought to determine the pharmacotherapeutic effect of the aqueous stem cell extract of *Malus domestica* (MD) in rats

Method: Antidiabetic effect of the aqueous extract of MD (50,100, 200, and 400 mg/kg) was investigated in normoglycemic and oral glucose-induced hyperglycemic rats that were fasted overnight. Furthermore, acute oral toxicity studies were conducted using the limit dose test of the Up-and-Down Procedure according to OECD/OCDE test guidelines on acute toxicity. In addition, α-amylase and α-glucosidase enzyme inhibitory assays, phytochemical analysis, and antioxidant activity were assessed.

Results: The phytochemical constituents exhibited flavonoids, phenols, phlobatannins, reducing sugars, carbohydrates, steroids, and cardiac glycosides**.** No mortality or signs of toxicity after oral administration with a single dose of 5 g/kg of the MD aqueous extract was recorded. Inhibition of α-amylase and α-glucosidase, positive antioxidant scavenging activity, and a significant ($p < 0.0001$) reduction in blood glucose level in the hyperglycemic-induced rats but not the normoglycemic were discovered. Pearson correlation showed an association between 50 and 100 mg/kg of MD and glibenclamide.

Conclusion : There could be a strong correlation between MD as a potential antidiabetic drug due to the antioxidant properties it possesses.

Keywords : Diabetes Mellitus, *Malus domestica*(*Borkh*), Swiss Apple, Pearson correlation Uttwiler Spätlauber

1.0 Introduction

The Swiss Apple {*Malus domestica* (*Borkh*)} of the Uttwiler Spätlauber specie, is a rare and nearly extinct variety with its main distribution in Thurgau, Switzerland [1].

Researchers have developed a technology enabling the application of the endangered apple cultivar in the cosmetic& aesthetic dermatology specialty as a novel anti-aging/anti-wrinkling breakthrough [2].

The use of the Swiss apple stems from the basis of reports that punctured unpicked apples or tree bark have the ability to heal themselves. Furthermore, the picked and uneaten ones last longer than the other species [3].

Diabetes Mellitus (DM) is a multi-systemic disorder that inflicts chronic debilitating complications leading to microvascular diseases (nephropathy, retinopathy, and neuropathy) and macrovascular diseases (stroke, coronary heart disease, and peripheral arterial disease) [4].

Current projections of the global prevalence of diabetes from 2019 with an estimate of 9.3 % (463 million) were expected to increase to 10.2 % by 2030 (578 million) with an upsurge to 10.9 % by 2045 (700 million) [5].

The prevalence is higher in urban (10.8%) than rural (7.2%) areas, and in high-income (10.4%) than low-income countries (4.0%) [6].

Although previously considered a disease in affluent nations, DM is now growing rapidly in developing nations including Sub-Saharan Africa [7]. This increasing prevalence is explained by acculturation, rapid and uncontrolled urbanization leading to a change in diet, lifestyle, obesity, and decreased physical activity [8].

Chronic complications in poorly treated cases result in neuropathy with increased risk of foot ulcers, nephropathy which is a major contributor to kidney failure, retinopathy with increased risk of loss of vision, lower limb ischemic disease with increased risk of foot amputation and vasculopathy that hastens the progression of cardiovascular disease [9-12].

The goal of the treatment modality for diabetes is therefore to reduce blood glucose levels as close to normal as safely possible because of its high rate of significant morbidity and mortality, poor quality of life, and reduced life expectancy. Recent studies have designated diabetes to be the third leading cause of death worldwide [13].

The therapeutic focus of preventing these complications according to conventional therapy is via the use of insulin or oral hypoglycemic agents such as metformin [14].

However, traditional hypoglycemic medications are not without their limitations of temporary therapeutic outcome versus long-term cost implications, hence the need for a treatment modality that will combat the significant burden that diabetes poses to these economies [15].

Furthermore, the use of routine anti-diabetic agents is a lifelong commitment that in Sub-Saharan Africa comes from personal finances, a daunting experience in a time when earning capacity is not commensurate with the expenses required for sustainability [16].

The adoption of this plant stem cell approach over the orthodox medical practice could offer insight into novel therapeutic options for blood glucose control. There are shreds of evidence that support the potential of the stem cell extract of *Malus domestica* (Borkh) to elucidate a novel therapeutic outcome in diabetes and other disease conditions [17].

Several results proposed that *Malus domestica* (Borkh) contains compounds with antioxidant activity, anti-inflammatory mechanisms, carcinogenesis, antimutagenicity, and antiproliferative effect [18]. The knowledge of using this approach could be effective in treating respiratory problems (i.e., asthma), diabetes mellitus, cardiovascular disease, and cancer prevention [19].

In spite of this, the hesitation in acceptance among health practitioners is a result of a lack of adequate information on the application of plant stem therapy in the clinical setting. This can be attributed to the paucity of research on its use as a successful remedy outside the common treatment as an anti-aging/anti-wrinkling agent. There will be wider acceptance if other sub-specialties adopt the use.

Plant stem cells refer to the undifferentiated totipotent cells located in the meristem of the plant responsible for the regeneration and repair of plant parts for self-renewal and self-growth purposing [20]. It employs the use of either stem cells with the multipotent ability and/or biological products that have the capacity to stimulate the transportation and proliferation of stem cells to the injured tissue thereby restoring function and repairing the damage [21].

Tissue repair is a complicated biologic process facilitated by the interplay of growth factors generated by the cells of the regeneration process upon reaching a proper concentration, causing the trigger to repair the defective tissue/organ [22].

Another school of thought that uses human stem cell therapy as an option in medical practice is not without its challenges [23]. The skepticism of this method stems from various factors such as religious, ethical & cultural beliefs, transplant rejection, and lifelong use of immunosuppressant therapy vs side effects of their use [24].

Nevertheless, a lot is yet to be explored in the endless possibilities that could arise from plant stem cell technology as a novel intervention in clinical practice as science is constantly evolving.

Hence, an understanding of the several pathways to provide adequate scientific evidence to explain the mechanism of action will ameliorate the doubt and encourage translational medicine; from bench to bedside in a clinical setting.

Thus, this study assesses the phytochemical effect and pharmacotherapeutic effect of the aqueous stem cell extract of Swiss apple {*Malus domestica* (Borkh)} in Diabetes Mellitus.

2.0 Materials and Methods

2.1 Extract preparation

This current study purchased extracts of apple PhytocellTec^{TM} (*Malus domestica*) stem cells from one of the Phyto science representatives in Nigeria. The product is marketed by Phyto Science Malaysia, and produced by a group of biotechnology researchers (Mirelle Biochemistry) in Zurich, Switzerland. It is a powder preparation, water-soluble/without alcohol, and free of preservatives. PhytocellTec ^{TM} *Malus domestica*(Borkh) is a patented liposomal preparation of apple stem cell extract derived from Uttwiler *spätlauber,* a rare species of Swiss apple administered via a sublingual route.

2.2 Chemicals

Ascorbic acid, glibenclamide, D- glucose, and all other chemical reagents were of analytical grade.

2.3 Animal study

Thirty rats (150- 200 g, 10 - 12-week-old) of the male sex were obtained from the Laboratory Animal Centre, College of Medicine, University of Lagos. Animals were allowed to acclimatize for seven days and were properly housed in transparent plastic padded with wood shavings under standard conditions: Ambient temperature (28±2) temperature, 55+-5% relative humidity, and standard photoperiod of 12h light: 12h dark cycle) prior to the commencement of the experiment. They were maintained on standard rodent pellets (Livestock Feeds PLC, Ikeja, Lagos, Nigeria), and provided with water*ad libitum.* The rats were handled in accordance with international principles guiding the use and handling of experimental animals [25]. The experimental protocol was approved and was in strict compliance with the Health Research Ethics Committee of the College of Medicine of the University of Lagos, Nigeria (CMUL/HREC/11/17/283).

2.4 Acute toxicity Study

Eight healthy albino male rats (10 - 12-week-old) weighing (140 - 200 g) were fasted overnight and employed for acute oral toxicity using the limit dose test of the Up-and-Down procedure was conducted in compliance with the OECD/OCDE Test Guidelines on Acute Oral Toxicity 420[25]. The experimental group of five rats was administered orally with a single dose of 5g/kg of aqueous *Malus domestica* (MD) extract while the control group of three rats was administered 10mg/kg of normal saline. The behavioral toxic effect (such as hyperactivity, hyperventilation, diarrhea, urination, and calmness), were recorded for the first 2 h and then, 24 hours post-treatment for mortality followed by 14 days for any signs of delayed toxic reactions or mortality.

2.5 Phytochemical analysis

The phytochemical constituent of MD extract was tested for the presence of saponins, tannins, alkaloids, flavonoids, anthraquinones, glycosides, and reducing sugars using simple and standard qualitative and quantitative methods described by Sofowora; Trease, and Evans [32,33]. The simple quantitative analysis of the extract was based on the intensity of the color change.

Briefly described are the tests of the qualitative phytochemical analysis of the aqueous *Malus domestica* (MD) extract determined as follows:

Alkaloids : A quantity (3 mL) of the concentrated extract was taken into a test tube and 1 mL HCl was added the mixture was heated gently for 20 minutes, cooled, and filtered. Into 2 mL of the cold-water extract in different tubes, were added 6 drops of different alkaloids reagents, namely: Dragendorff's, Mayer's, or Wagner's reagent. The presence and colors of any precipitate were noted. Creamish precipitate or brownish-red precipitate or orange precipitate indicated the presence of respective alkaloids.

Saponins : - Using the frothing test, the aqueous extract was obtained by boiling it in a water bath. The extract was transferred into a test tube and shaken vigorously then left to stand for 10 minutes and the result was noted. Frothing persistence meant saponins were present.

Steroids : Salkowski's test: The extract (200mg) was dissolved in 2 mL of chloroform. Concentrated tetraoxosulphate (VI) acid was carefully added to form a lower layer. A reddish-brown at the interface indicated the presence of a steroidal ring. In this test, 2 mL of acetyl anhydride was added to 0.5 g of an extract with 2 mL concentrated H_2SO_4. The color change from violet to blue or green is an indication of steroids.

Flavonoids : The powdered sample (200mg) was boiled in 10 mL of absolute ethanol for 10 minutes. The solution was allowed to cool and then filtered. To 2 mL of the filtrate was added concentrated HCl and magnesium ribbon. Pink-tomato red color indicated the presence of flavonoids.

Tannins : The aqueous extract (200mg) was dissolved in 10 mL of distilled water and then filtered. A 2 mL of filtrate was pipetted into a test tube after which 2 mL of 15% $FeCl_3$ was added. The color change was observed. The Blue-black presence indicated the presence of tannins.

Phlobatannins : The deposition of a red precipitate when the aqueous extract of each plant sample is boiled with 1% aqueous HCl was taken as evidence for the presence of phlobatannins.

Anthraquinone : Bontrager's test was done where chloroform extract of the powdered sample was obtained by boiling in the water bath. To 2 mL of this extract hydrolyzed with dilute H_2SO_4, 1 mL of dilute (10%) ammonia and benzene was added and the mixture was shaken. The formation of pink coloration suggested its presence.

Cardiac glycoside : Keller-Kiliani test for de-oxy sugars in cardiac glycosides was performed. A methanol extract was obtained and the extract was reduced to dryness and 50 mg of this was dissolved in 2 mL of chloroform. Tetraoxosulphate (VI) acid was added to form a layer and the color at interphase was recorded.

Reducing sugar : Using Fehling's procedure, water extract of the powdered material was obtained by boiling it in a water bath. Into each test tube containing 2 mL of the extract, 1 mL of Fehling's solutions A and B were added. The mixture was shaken and heated in a water bath for 10 minutes. The red precipitate color obtained was recorded.

Briefly described are the tests for the quantitative phytochemical analysis of the aqueous *Malus domestica* (MD) extract determined as follows.

Total Steroids

The solution of 1mL of aqueous *Malus domestica* (MD) extract was transferred into a 10 mL volumetric flask. In addition, H_2SO_4 (4N, 2 mL) and $FeCl_3$ (0.5% w/v, 2 mL), was added, followed by $K_3[Fe\ (CN)_6$ solution (0.5% w/v, 0.5 mL). The mixture was heated over a water bath at 70 +- 2^0 C for 30 minutes with occasional shaking. The volume was made up to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank. [34]

The total steroid was calculated using the formula below:

Weight of sample (g): Absorbance of sample/Absorbance of standard x Concentration of standard

Percentage % Yield = Weight of saponin / Weight of sample x100

Total Cardiac Glycosides

In this test, 10% of aqueous *Malus domestica* (MD) extract was mixed with 10 mL of freshly prepared Baljet's reagent (95 mL of 1% picric acid + 5 mL of 10% NaOH). The mixture was allowed to stand for 1-hour. In addition, there was a dilution with 20 mL of distilled water and the measurement of absorbance at 495 nm using a UV spectrophotometer. [35]

The percentage yield of cardiac glycosides was calculated as follows:

Weight of sample (g): Absorbance of sample/Absorbance of standard x Concentration of standard.

Percentage % Yield = Weight of saponin / Weight of sample x100

Reducing Sugar Content

The reducing sugar content (RSC) was determined using the 3,5-dinitrosalicylic acid (DNSA) method. The measurement was performed according to the procedure of Krivorotova and Sereikaite [36] with slight modification. DNSA reagent was prepared by dissolving 1 g of DNSA and 30 g of sodium-potassium tartaric acid in 80 mL of 0.5 N NaOH at 45deg C. Following dissolution, the solution was cooled to room temperature and diluted to 100 mL using distilled water. Subsequently, 2 mL of DNSA reagent was pipetted into a test tube containing 1 mL of plant extract (1 mg/ mL) and kept at 95deg C for 5 min. After cooling, 7 mL of distilled water was added to the solution and the absorbance of the resulting solution was measured at 540 nm using a UV-VIS spectrophotometer (Shimadzu UV-1800). The reducing sugar content was calculated from the calibration curve of standard D-glucose (200-1000 mg/L), and the results were expressed as mg D-glucose equivalent (GE) per gram dry extract weight.

Total Flavonoids Content

The total flavonoid content (TFC) was determined as previously described by Yoo *et al* [37]. In this assay, 1 mL of extract (1.5 mg/ mL) was added to 4 mL of distilled water and 0.3 mL of 5% (w/v) sodium nitrite. After 5 min of reaction, 0.6 mL of 10% (w/v) $AlCl_3$ was added, and 6 min later, 2 mL of 1 M NaOH and 2.1 mL of distilled water were added. Absorbance was read at 510 nm. The total flavonoid content was determined in triplicate and expressed as milligrams of quercetin equivalents (QE)/g of extract.

Total Phenols Content

The determination of the total phenols content (TPC) employed the Folin-Ciocalteu method [39]. A 100 µL of extract (1.5 mg/ mL) was mixed with 2 mL of water along with 1 mL of

Na $_2CO_3$ 15% (w/v) aqueous solution, and 0.2 mL of Folin-Ciocalteu reagent. After 2 h of incubation at 25 *C, the absorbance was measured at 765 nm using a UV-vis Jenway 6003 spectrophotometer (Milan, Italy). TPC was determined in triplicate and expressed as milligrams of chlorogenic acid equivalents (CA)/g of extract. A combination of 1 mL of extract, 1 mL of distilled water and 5 mL of the anthrone reagent were added and standard mixing was done by vortexing. The tubes were cooled and later covered for incubation at 90^0C for 17 minutes or boiling water bath for 10 minutes. The room temperature was cooled and measured at 620 nm against a reagent blank [40].

2.6 Oral glucose tolerance test

Acute extract treatment in oral glucose-induced hyperglycemic rats

This assay according to a method by Adeneye and Adeyemi [41] was employed with slight modification. In the high oral glucose hyperglycemic model, 12 - 14 hr fasted rats were randomly allotted to six groups (n= 5) such that the difference within and between groups does not exceed ±20% of the average weight of the sample population of rats. Group I rat, which served as the untreated control, were orally pretreated with 10 mL /kg of distilled water 1 hr before treatment with another 10 mL /kg/oral of distilled water. Group II rats served as the model control and were pretreated with 10 mL /kg/oral distilled water 1 hr before oral treatment with 2 g/kg of D-glucose (Analar). Groups III-VI rats which served as the treatment groups were orally pretreated with 5 mg/kg of glibenclamide (Daonil®, Hoechst Marion Roussel Limited, Mumbai, India), 50 mg/kg, 100 mg/kg, and 200 mg/kg of MD, respectively, for 1 hr before the oral administration of 2 g/kg of D-glucose. Blood sample from the rat tail vein for fasting whole blood glucose was collected by tail tipping method. The tail was gently squeezed to let out 2 - 3 drops of fresh whole blood which were placed on the test spot of the glucose strip after which the test strip is gently inserted into the Test Strip

Platform of the Microprocessor digital blood glucometer and the readings were recorded [42]. blood glucose concentrations were determined at 0 hr, 30min, 60min, 90min, and 120min, respectively

Repeated extract treatment in normoglycemic rats

The assay adopted is the same as the earlier reported protocol. In the repeated dose model, 12 - 14 hr fasted rats were randomly allotted to six groups (n=5) such that the difference within and between groups does not also exceed ±20% of the average weight of the sample population of rats. Group I rat, which served as the untreated control, were orally treated with 10 mL /kg of distilled water while Groups II-VI served as the treatment groups, were treated with single, daily oral 50 mg/kg, 100 mg/kg, 200 mg/kg, 400 mg/kg and 5 mg/kg of glibenclamide (Daonil®, Hoechst Marion Roussel Limited, Mumbai, India) of MD for 14 days, respectively. The fasting blood glucose concentrations were determined on the 1st and 15th day of the experiment following an overnight fast.

2.7 *In vivo* antidiabetic activity

Alpha-amylase inhibition assay

The assay was carried out using the 3,5-dinitrosalicylic acid (DNSA) method with slight modification [26]. The assay mixture comprised 500 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing 6 mM sodium chloride (NaCl), 0.05 units of α-amylase solution and aqueous*Malus domestica* (MD) extract at concentrations of 20, 40, 60, 80 and 100 µg/ mL (w/v). Acarbose at the same concentrations as MD was used as the positive control. Each concentration was assayed in triplicate. The assay mixture was initially incubated at 37 °C for 25 min. After incubation, 250 mL of 0.5 % (v/v) starch solution in the abovementioned buffer was added to the tubes and incubated for 20 min at 37 °C. The reaction was stopped by adding 1 mL of DNSA reagent and then incubated in a water bath for 10 min. The tubes were cooled and the absorbance was measured at 540 nm (T80 Spectrometer, PG Instrument Ltd, Leicestershire, United Kingdom). The tube with α-amylase but without MD served as the control with 100 % enzyme activity.

Alpha-glucosidase inhibition assay

The assay was carried out with slight modifications using α-glucosidase obtained from Saccharomyces cerevisiae [27]. The assay mixture consisted of 150 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 6 mM NaCl, 0.1 U of α-glucosidase and MD at concentrations of 20,40,60,80 and 100 µg/ mL (w/v). Acarbose at the same concentrations as MD was used as the positive control. The mixture was initially incubated at 37 °C for 15 min. After incubation, 50 mL of 2 mM para-nitrophenyl α-d-glucopyranoside in 0.1 M sodium phosphate buffer was added to the mixture and incubated at 37 °C for 25 min. The reaction was stopped by adding 50 mL of 0.1 M sodium carbonate (Na_2CO_3). The absorbance was measured at 540 nm (T80 Spectrometer, PG Instrument Ltd, Leicestershire, United Kingdom). The tube with α-glucosidase but without MD served as the control with 100 % enzyme activity. The formula below was used to calculate percentage inhibition for both α-amylase and α-glucosidase inhibitory assays:

Percentage inhibition (%) = *Absorbance of control - Absorbance of MD* × 100

Absorbance of control

2.8 Antioxidant activity

2.8.1 DPPH radical scavenging activity assay

The free radical scavenging activity of MD extract was estimated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to a procedure described by Cuendet *et al* [28]. An aliquot (0.5 mL) of MD in ethanol (95 %) at different concentrations (25, 50, 75, 100 µg/ mL) was mixed with 2.0 mL of reagent solution (0.004 g of DPPH in 100 mL methanol). The control contained only DPPH solution in place of the sample while methanol was used as the blank. The mixture was vigorously shaken and left to stand at room temperature. After 30 min, the absorbance of the test mixture was read at 517 nm. The scavenging effect was calculated using the expression:

% inhibition = $[A_{b0}$ - $Ab_1]$ x 100

Abo

where Abo is the absorbance of the blank sample and Ab1 is the absorbance of the extract.

2.8.2 Reducing Power Assay

The reducing power was determined according to an established procedure earlier described by Cuendet *et al* . Various concentrations of MD extracts (25, 50, 75, and 100 ug/ mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Subsequently, 2.5 mL of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 650 rpm for 10 min. The upper layer was mixed with 2 mL of deionized water and 1 mL of 0.1 % of ferric chloride, and the absorbance was measured at 517 nm.

2.8.3 Estimation of Total Phenolic Content

This assay according to a method by Wolfe *et al* . [30] was employed with slight modification using a reference standard (gallic acid). The extract (0.5 mL) was added to the standard reagent (Folin- Ciocalteu; 0.1 mL; 0.5 N); mixed and incubated for 15 min. In addition, sodium carbonate solution (2.5 mL; 7.5% w/v) was added and the mixture was further incubated for 30 min at room temperature. The absorbance was spectrophotometrical1y estimated at 760 nm. The gallic acid equivalent (GAE) (mg/g of dry mass) was used to express the concentration of total phenolic content.

2.8.4 Estimation of Total Flavonoid Content

The slightly adjusted method described by Chang *et al* . [31] was used to estimate total flavonoid content. Quercetin was used as a reference standard and the result was expressed as total quercetin equivalent (QE). mL (1 mL; 100 µg/ mL) was mixed with methanol (3 mL), 10% $AIC1_3$ (0.2 mL), and potassium acetate (0.2 mL: 1 M). The mixture was incubated at room temperature for 30 min, after which the absorbance of the mixture was measured at 415 nm.

2.9 Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM) and analyzed in two ways: Pandas, Python 3.6 to determine Pearson correlation with statistical significance set at $p < 0.001$ and post hoc test for comparison of the mean values among the multiple groups using two-way ANOVA followed by Tukey's *post hoc* test on Graph-Pad Prism 6 (Graph-Pad Software Inc., California, United States of America). Statistical significance was considered at $p < 0.05$.

3.0 Results

3.1 Phytochemical analysis

Results of the phytochemical studies showed MD contains phlobatannins, phenols, steroids, flavonoids, carbohydrates, cardiac glycosides, and reducing sugars which were determined by simple and standard qualitative methods and shown in Tables 1 and 2. However, there was an absence of terpenoids, saponins, and tannins. Table 2 shows the quantitative analysis showed the quantity exhibited in the MD extract.

3.2 Acute toxicity

Table 3 shows the MD- pretreated mice with a single oral dose of 5g/kg p.o exhibited the following behaviors in the initial 30-minutes of the acute toxicity procedure: an initial agitation then calmness, a rapid-onset increased somatomotor activity (shivering, climbing, rearing, volleying, head-dip immobility), tachypnea, sedation, abdominal writhing, bilateral narrowing of the eyelids, tremor, and piloerection, sniffing, hurdling together and drowsiness. However, this did not result in overt toxic symptoms or death in both the MD-treated rats or the control groups. After 24 h post-treatment, no signs of toxicity and mortality were recorded. The rats neither showed any signs of delayed toxicity nor mortality after 14 days of observation. The rats in the normal saline pretreated group (control group) showed neither immediate or delayed abnormality (after

14days) in behaviors nor mortality. The LD_{50} estimate from the AOT425Pgm program was greater than 5g/kg of body weight/oral route.

3.3 *In vivo* antidiabetic activity

3.3.1 Alpha-glucosidase inhibitory assay

Malus domestica (MD) extract produced 32.76 %, 38.90 %, 54.96 %, 65.83 %, and 78.90 % for the first assay and the 31.65%, 38.27%, 52.28%, 66.14%, and 79.37% inhibition of α-glucosidase activity for the second assay at 20, 40, 60, 80 and 100 µg/mL concentrations respectively from Tables 4 and 5. The IC_{50} value generated for MD was 31.55 µg/mL (Figure 1). The highest percentage inhibition of the α-glucosidase enzyme was displayed at 100 µg/mL.

The standard drug, acarbose, exhibited 46.53 %, 51.47 %, 58.87 %, 67.92% and 83.20% for the first assay and then 46.42 %, 51.70 %, 59.11 %, 67.57% and 82.96% inhibition of α-glucosidase enzyme activity for the second assay at the same concentrations as MD, respectively. The IC_{50} value generated for acarbose was 20.08 µg/mL (Figure 2).

3.3.2 Alpha-amylase inhibitory assay

Malus domestica (MD) extract produced 25.91 %, 41.78 %, 53.76 %, 60.86 %, and 70.61 % for the first assay and the 26.74%, 40.95%, 26.60%, 59.75%, and 71.03% inhibition of α-glucosidase activity for the second assay at 20, 40, 60, 80 and 100 µg/ mL concentrations respectively from Tables 6 and 7. The IC_{50} value generated for MD was 29.34 µg/ mL (Figure 3). The highest percentage inhibition of the α-amylase enzyme was displayed at 100 µg/ mL

The standard drug, acarbose, exhibited 51.46 %, 64.86 %, 74.50 %, 81.20 %, and 87.90% for the first assay and then 52.19 %, 64.04 %, 73.80 %, 80.96 %, and 88.25 % inhibition of α-glucosidase enzyme activity for the second assay at the same concentrations as MD, respectively. The IC_{50} generated for acarbose was 16.84 µg/ mL (Figure 4).

3.4 *In vitro* antioxidant assay, total phenolic and total flavonoid contents

Malus domestica (MD) extract displayed a comparable antioxidant effect with respect to ascorbic acid which was used as the reference as seen in Tables 8 -10. The IC_{50} values of the extract were also established in Figures 5a,5b and 5c (25.5, 20.9 and 35.5mg/mL respectively). The total phenolic, antioxidant capacity, and flavonoid contents were estimated to be 414.9 ±0.02 mg GAE/g dry weight, 36.93 mg/100g, and 328.8 ±0.01 mg QE/g dry weight respectively as seen in Table 11.

3.5 Oral glucose tolerance test

3.5.1 Acute extract treatment in oral glucose-induced hyperglycemic rats

From figure 6, a single high oral glucose treatment was associated with significant ($p < 0.0001$) hyperglycemia, particularly within the first 90min and 120min post-administration. As shown in the same figure, the glibenclamide administered at 5mg/kg significantly reduced blood glucose level (BGL) at all the time intervals but with varying p-values: 30min ($p< 0.01$) 60min ($p < 0.05$), 90min, and 120min ($p< 0.0001$) compared to the glucose treated group. This group also significantly ($p<0.001$) reduced the BGL at all time intervals compared to the distilled water (DW) treated group (negative control). The aqueous extract of the MD pretreated rats administered orally (50-200mg/kg) did alter the BGL significantly compared to the untreated DW group. Over the succeeding 90min up to 120min, the 50 - 200 mg/kg of MD pretreated rats significantly reduced ($p < 0.0001$) the BGL compared to the glucose-treated group. However, 200mg/kg of MD showed a significant ($p<0.0001$) difference in the BGL at 30 min, 60 min, and 90 min comparable to that of glibenclamide. Similarly, 50 and 100 mg/kg of MD showed significant differences at 30 min ($p<0.001$), 60 min, and 90 min ($p<0.0001$) comparable to that of glibenclamide. At 120 min, the 100 and 200 mg/kg of MD had a significant effect ($p< 0.05$ and $p< 0.001$ respectively) compared to glibenclamide.

3.5.2 Repeated extract treatment in normoglycemic rats

From figure 7, the repeated dose study of normoglycemic rats on day 15 showed the glibenclamide-treated group had a significant (p=0.0005) hypoglycemic effect compared to the DW (untreated) group. However, the aqueous extract of MD at 50 - 400 mg/kg/day did not have a significant hypoglycemic effect when compared to the untreated group. Only the aqueous extract of MD at 200 mg/kg and 400 mg/kg/day of MD had a significant effect ($p = 0.003$ and $p < 0.0001$) when compared with the glibenclamide group.

As depicted in Figure 8, a two-tail Pearson correlation analysis was used to test the bivariate linear relationships among all the various groups. Mean fasting BGL of MD 50mg/kg and 100 mg/kg demonstrated a high negative correlation (r (28) = -0.75; r (28) = - 0.79) to the glucose-treated group, MD 200 mg/kg and distilled water group showed negligible correlation (r (28) = -0.18, r (28) = +0.24) while the glibenclamide group expressed low negative correlation (r (28) = -0.41) to the glucose treated group.

Mean fasting BGL of MD at 50 mg/kg and 100 mg/kg were lowly positively correlated to glibenclamide (r (28) = 0.42; r (28) =0.33), 200 mg/kg was moderately negatively correlated (r (28) = -0.53) with glibenclamide while DW has a high negative correlation (r (28) =0.82), with glibenclamide. There was a strong positive correlation between the MD 50 and 100mg/kg doses respectively (r (28) =0.95, $p < 0.001$).

4.0 Discussion

According to WHO, globally the prevalence of diabetes has been increasing steadily with a current population of 420 million and directly responsible for 1.5 million deaths each year[43] .The goal of the treatment modality for diabetes is to reduce blood glucose levels as close to normal as safely possible because of its increased risk of micro and macrovascular complications. However, conventional anti-diabetic therapy is not cost-effective due to life-long use and frequently reported drawbacks and attendant complications such as hypoglycemia, weight gain, gastrointestinal disturbance, lactic acidosis, and fluid retention[44].

Hence, the unending pursuit of a novel treatment for diabetes using plant stem cell therapy which has great potential in achieving optimal glycemic control is a welcome development. However, it is common knowledge from research that various inhibitors of carbohydrate hydrolyzing enzymes into monosaccharides by α-amylase and α-glucosidase hence delaying glucose absorption, have been used as oral hypoglycemic agents (such as acarbose) in Type 2 diabetes[45].

From our study, the *Malus domestica* (MD) extract showed inhibition of these enzymes' maximum at 100 µg/MD concentrations.

A study by Xiao *et al* [46] discovered that the inhibitory property of polyphenolic components isolated from Apples *(Malus domestica)* on α-amylase and α-glucosidase enzymes was similar to that of acarbose, although acarbose had a higher inhibitory effect on α-amylase.

Furthermore, the phytochemical studies established the presence of phlorotannins, phenols, steroids, flavonoids, carbohydrates, cardiac glycosides, and reducing sugars in *Malus domestica* (MD) aqueous extract. The presence of these compounds could account for the significant anti-oxidant effect of the extract, either alone or in synergy with one another.

It is well documented from the literature that the aforementioned naturally occurring biological compounds in plants possess hypoglycemic and anti-inflammatory functions[47].

Flavonoid compounds which represent a class of phenolic compounds have been reported to regenerate pancreatic β-cells and modulate insulin secretion [48].

The dose-dependent antioxidant activity of MD was established using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, lipid peroxidation and reducing power was also assessed. This effect was maximal at 100µg/MD and similar when compared to ascorbic acid. The IC_{50} values of the extract were within the range of 10-50mg/mL which according to Jadid *et al* [49] demonstrates strong antioxidant activity.

As earlier mentioned, MD aqueous extract contained naturally occurring phenolic compounds such as flavonoids from the phytochemical study, that have been reported to have antioxidant characteristics [50].

The radical scavenging action by chelating metal ions and/or the antioxidant effect of flavonoids is mediated by the functional hydroxyl groups it possesses [51]. The well-known medicinal efficacy of flavonoids is due to their anti-bacterial, anti-inflammatory, anti-viral, anti-cancer, and hypoglycemic functions [52].

Khan *et al* [53] revealed that the pathogenesis of diabetes mellitus and oxidative stress are closely related, thus causing the release of free radicals. The simultaneous depletion by antioxidant defense mechanisms is the target goal of therapy to prevent deleterious complications.

Carbohydrate metabolism releases glucose as an end product that acts as a fuel for the brain activity [54]. Glucose levels in the blood must be constantly maintained within a physiological range (fasting 70–80 mg/dL and postprandial 140–160 mg/dL) to provide fuel for vital tissues (such as the CNS) and sustain anabolism [55].

This was comparable to our study where the fasting blood glucose level in this data set was between 73-117 mg/dl. In addition, a normal physiologic response was observed post administration within the first 90min and 120min in the single oral glucose-treated rats, where significant ($p < 0.0001$) hyperglycemia at 90 min (postprandial value 135-190 mg/dL) and at 120 min (postprandial 161- 193 mg/dl) was observed.

The glibenclamide treatment group was associated with a significant reduction of blood glucose level compared with the glucose-treated group ($p < 0.01$) and distilled water-treated group (negative control) ($p<0.001$) from 30min till the end of the experiment.

Glibenclamide is in the sulfonylurea class of drugs that stimulates insulin secretion to reduce blood glucose levels[56].

From our study, the aqueous extract of the 50 - 200 mg/kg of MD pretreated rats significantly reduced ($p < 0.0001$) the BGL compared to the glucose-treated group though did not cause significant alteration compared to the negative control.

This was similar to a study by Patocka *et al* where the leaf extracts of *Malus domestica* reduced fasting blood glucose levels in alloxan-induced hyperglycemic rats [57]. Our study also demonstrated that the aqueous extract of the 50 - 200 mg/kg of MD had a similar effect on BGL comparable to glibenclamide at different time intervals although after 120min only the 100mg/kg and 200mg/kg of MD had comparable effects.

It is noteworthy from the literature that the flavonoid content of apple juices which is the biological source of *Malus domestica* (Swiss apple) was found to affect insulin, glucose-dependent insulinotropic polypeptide, and glucagon-like peptide-1 in volunteers which resulted in delayed intestinal absorption of glucose [58].

Our study demonstrated in the repeated dose study of normoglycemic rats on day 15 that the glibenclamide-treated group had a significant ($p=0.0005$) hypoglycemic effect compared to the DW (untreated) group. The aqueous extract of MD at 50 - 400 mg/kg/day did not have a similar effect. However, only the aqueous extract of MD at 200mg/kg and 400mg/kg/day of MD had a significant effect ($p = 0.003$ and $p < 0.0001$) when compared with the glibenclamide group.

This shows that the extract probably does not have an effect on normoglycemic rats.

Further analysis to corroborate an association between glibenclamide versus MD extract and also to confirm the hypoglycemic effect by the relationship with the glucose-treated group was achieved using Pearson correlation analysis. This could be a possible pointer as to similarity in the mechanism of action with sulphonylurea subclass (using glibenclamide as standard) and also show hypoglycemic effect.

It was demonstrated that the MD 50 mg/kg and 100 mg/kg demonstrated a strong negative correlation with the glucose treatment group. This means that there was an inverse relationship between the groups though it was not statistically significant.

The MD 200mg/kg on the other hand with the distilled water group showed negligible correlation (not statistically significant). The MD at 50 mg/kg and 100 mg/kg were lowly positively correlated to glibenclamide while the 200 mg/kg was moderately negatively correlated (not statistically significant).

There was a statistically significant, strong positive correlation between the doses of MD at 50 and 100 mg/kg ($p < 0.001$). This shows there was a hypoglycemic effect in the 50 and 100mg/kg doses (strong correlation) and there was some association with glibenclamide although further studies to assess the exact mechanism of action of*Malus domestica* will have to be further investigated.

From our study, the MD had a similar effect with both alpha-glucosidase inhibitor (acarbose) and sulphonylurea (glibenclamide) subclass of antidiabetic drugs. However, further studies after experimental induction of diabetes to confirm the hypoglycemic mechanism in MD-pretreated rodents which is currently ongoing in our research laboratory is recommended.

5.0 Conclusion

The results obtained from this study demonstrated that the aqueous extract of Swiss Apple {*Malus domestica* (*Borkh*)} of the Uttwiler Spätlauber specie has an inherent prospect of achieving optimal glycemic control based on its components and activities. The effect was seen in hyperglycemic but not normoglycemic rats. It is possible that the anti-oxidant property it possesses is a result of the phenolic constituents identified and the mechanism of action may be similar to glibenclamide/acarbose. This justifies that plant stem cell therapy may be used in treating diabetes that is caused by oxidative stress. Our study has formed a template that can constitute areas for future studies on the hypoglycemic effect in MD- pretreated experimental rodents.

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Authors' contributions

IAA conceived the study concept and worked on the study design, data collation, analysis, and preparation of the manuscript. OCP and INF participated in study design, and data collection while SMF analyzed the data. EOA and IAO were the supervisors of the project. All authors read and approved the final manuscript.

Conflict of interest

None declared.

Consent for publication

"Not applicable

Ethics approval

The ethics approval was granted for this study by the Health Research Ethics Committee (HREC) of Lagos University Teaching Hospital with reference No: CMUL/HREC/1106/19. This study complied with all institutional guidelines and regulations as regards ethics and permission for the research.

Availability of data and materials

The datasets during and/or analyzed during the current study are available in the additional supporting files

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