Pharmacological Research 212 (2025) 107602

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Phenolic acids from *Anisopus mannii* modulates phosphofructokinase 1 to improve glycemic control in patients with type 2 diabetes: A double-blind, randomized, clinical trial☆

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ARTICLE INFO

Keywords:
Diabetes
Phosphofructokinase 1
Randomized trial
HbA1c
Anisopus mannii

ABSTRACT

Phenolic acid-rich fraction from *Anisopus mannii* (PhAM) contains abundance of ferulic acid, gallic acid, protocatechuic acid, and syringic acid. Among other glycolytic enzymes, in vitro, PhAM counteracted the binding of sodium orthovanadate to phosphofructokinase 1 (PFK-1), improving its activities. In a rat model of diet-induced diabetes, PhAM monotherapy reduced HbA1c by an average of 0.63 % and fasting plasma glucose by 25 mg/dl. This herb rescued β-cells from streptozotocin-mediated destruction, thereby improving glycemic control. Supported by the preclinical trial, eighty-five patients with type 2 diabetes (T2D) receiving first-line medications were enrolled in a double-blind, randomized, placebo-controlled trial with a 90 % power level. Patients were randomized into a placebo group or either of the following two treatment groups: oral administration of 12 mg or 20 mg/kg body weight of PhAM once every 48 h for 6 months. Both treatments were well tolerated. At the endpoint, more than 70 % of patients achieved a 0.5 – 2.0 decrease in HbA1c levels and a > 20 mg/dl decrease in fasting blood glucose, meeting the pre-specified primary outcome. 66 % of patients treated with 20 mg PhAM achieved the < 7 % HbA1c and HOMA-IR of > 1.0 goal. respectively. Our study shows that PhAM can supplement first-line medications to achieve target glycemic control within 6 months.

1. Introduction

Currently, many popular anti-hyperglycemic monotherapies are becoming less effective at achieving the glycemic goals in patients with type 2 diabetes (T2D) [1,2]. Due to the complexities and complications associated with diabetes, more patients require additional treatment plans to maintain long-lasting and stable glycemic control even when the glycemic goals are achieved in the interim[3]. Metformin is the most preferred first-line medication for T2D.[4]2018 Clinical Practice Guidelines Committees, Can. J. Diabetes 42 A6–A16.2018 Clinical Practice Guidelines Committees. Can J Diabetes 42, A6–A16 [4]. Metformin suppresses hepatic glucose output as the primary mechanism of

☆ Pan African Clinical Trial Registration number: PACTR202206531545729.

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https://doi.org/10.1016/j.phrs.2025.107602
Received 31 October 2024; Received in revised form 31 December 2024; Accepted 13 January 2025
Available online 14 January 2025

its anti-hyperglycemic effects[5]. However, the progressive deterioration of β-cell function in diabetes often causes loss of efficacy of metformin monotherapy, necessitating the administration of additional anti-hyperglycemic medications[6,7]. The FDA has approved several combination treatment plans for diabetes involving other drugs with various mechanisms of action. However, achieving target glycemic levels remains an unmet goal in the treatment of T2D, as approximately 537 million adults worldwide still have diabetes[8]. This clearly means that alternative treatment options are long overdue.

The use of naturopathic medicinehas emerged as a promising option for the management of diseases with complex pathological features, such as T2D[10-12,9]. Many current orthodox drugs are derived from herbal medicines. Metformin dates back to the use of *Galega officinalis* (goat's rue or French lilac) in medieval Europe to treat symptoms of diabetes[13]. Nonetheless, most of the popular herbal medicines have not yet gained recognition in orthodox care because research on these herbs is often carried out by researchers with infrastructural limitations to attain convincing scientific rigor required to gain global recognition. Herbs contain well-characterized bioactive components, and unlike orthodox monotherapies, the synergistic potential of several bioactive constituents of these herbs can be harnessed to provide better efficacy in diseases with multiple aetiologies, such as T2D.

Increasing evidence shows that the anti-hyperglycemic activity of various herbs is closely related to their insulin-secretagogue activities [14-16]. However, most of these studies fail to advance into clinical trials because they do not identify which bioactive fractions or compounds elicit these anti-hyperglycemic effects or their underlying mechanisms of action.

Anipsopus mannii, popularly known as the "diabetes killer" in Nigeria, deserves recognition for its therapeutic benefits. The flowering species grows in tropical environments in central Africa, and is a renowned traditional Nigerian medicine applied for its potent anti-hyperglycemic effect[17-19]. Various parts of the herb, including the stem, leaf, and bark are traditionally used for the management of diabetes. The stem extract reportedly produces glucose-lowering effects in both alloxan and streptozotocin-induced diabetes, while the leaves have well documented insulin-sensitizing effects in chemical models of preclinical diabetes[20-22]. Some bioactive compounds isolated from this herb have shown various biological activities[23]. 1,7-naphthyridine alkaloid, otherwise known as Bisleuconothine A 54, induced cell cycle arrest in various cancer cells while other major components, the 1-Dehydro-6-Gingerdione and ferulic acid reportedly enhances insulin secretion[24-26]. Phytol, an acyclic diterpenoid with well documented insulin-sensitizing potentials, was among the abundant phytochemicals components of A. mannii[27,28]. Manosrin, a novel phytochemical from A. mannii also produced glucose-lowering effect on alloxan-induced model of diabetes[21]. Despite the accumulating evidence suggesting that *A. mannii* produces potent anti-diabetic effects, its mechanism of action remains unidentified thereby affect its clinical integration.

This is the first study to show that specific phenolic acid fractions from this herb serve as an agonist for phosphofructokinase 1 (PFK-1), among other possible mechanisms, which led to significant glycemic control. We further identified that the phenolic fraction of this herb contains therapeutic doses of ferulic acid, gallic acid, protocatechuic acid, and syringic acid. PFK-1, an allosteric enzyme controlled by multiple activators and inhibitors, is the rate-limiting enzyme of the glycolytic pathway[29]. This means that the activity of PFK-1 directly controls glucose uptake and breakdown, and indirectly regulates other pathways involved in glucose metabolism.

Several drugs developed to target enzymes of the glycolytic pathway have shown promising efficacy in improving glucose sensitivity in patients with non-insulin dependent diabetes mellitus (NIDDM)[30,31]. Small molecule activators of glucokinase are popular drugs in this category and are well studied for their add-on effects on insulin, glucagon, and incretins, thereby improving the maintenance of glycemic homeostasis in patients with T2D[32-34]. Although the anti-hyperglycemic effects of targeting glycolytic enzymes are promising, the druggability of PFK-1 has not been fully studied, resulting in a paucity of literature on the efficacy of PFK-1 agonists in T2D. This study provides the preclinical and clinical evidence to encourage the druggability of PFK-1 for a more potent glycemic control in T2D patients. Thus, we presented a proof of concept that combination therapy of PhAM and metformin achieves better glycemic control than metformin monotherapy in T2D patients.

2. Methodology

Detailed procedures for extraction, fractionation, HPLC analysis, chromatograms, and readout of the *Anisopus mannii* (AME) are presented in the supplementary file.

2.1. Preclinical study

Wistar Albino rats were subjected to both chemical (alloxan (ALX) and streptozotocin STZ)) and diet (High Fat Diet (HFD)) models of diabetes, as defined in the sections below.

All animal handling and techniques occurred in accordance with the ARRIVE guidelines (https://arriveguidelines.org) and the National Research Council's Guide for the Care and Use of Laboratory Animals, approved by the Imo State University Ethics Review Board with ethical approval number, IMSU/BCM/ETS/20181219. Sex-matched Wistar rats (10–11 weeks old), weighing 150–200 g each, were purchased from the Department of Pharmacology, University of Port Harcourt, and housed at the Animal Facility of the Imo State University, Nigeria. Rats were housed in standard cages (maximum 4 rats per cage) with a 12-h light/dark cycle at 50 ± 5 % humidity and 23 ± 1 °C and provided with standard rat chow (ENVIGO-Harlan Teklad Global Rat Food Pellets; TD. 2018c, 18.4 % crude protein, 6 % fat, 44.2 % carbohydrate, and 3.1Kcal/g gross energy) *ad libitum* and saline unless otherwise stated. Food was withdrawn when required. A brief description of the experimmental groups are as follows:

Control – Various controls, normal and negative controls were used. For the chemical models of diabetes, the control was normoglycemic rats administered with rat chow and appropriate vehicle. A negative control was used for the diet model, which was rats fed with HFD without any treatment.

ALX – rats made diabetic with a single i.p. alloxan injection as described below

STZ – rats made diabetic with a single i.p. STZ injection as described below

ALX+AME – Alloxan induced diabetic rats treated with 100 mg/kg b.w aqueous extract of AMEALX+STZ – streptozotocin induced diabetic rats treated with 100 mg/kg b.w aqueous extract of AME

HFD – rats fed on high fat diet for 12 weeks as described below

HFD+PhAM - rats fed on high fat diet for 12 weeks and treated with 150 mg/kg bw of PhAM as described below

2.1.1. Induction of diabetes

Diabetes was induced after an 8 h fasting at 10 weeks of age with a single intraperitoneal (i.p) dose of 150 µl of freshly prepared 150 mg/kg b.w alloxan monohydrate (ALX) or streptozotocin (STZ), using sodium citrate as vehicle (10 mM, pH 4.5). The vehicle was applied to all rats, and rats receiving alloxan were housed separately from rats receiving streptozotocin. 72 h post injection, diabetes was confirmed and only rats with fasting blood glucose > 160 mg/dl were randomly assigned to the diabetes group. Each experimental group had 6 rats. For the treatment group, freshly prepared 100 mg/kg b.w aqueous extract of AME was administered orally or intraperitoneally once every 72 h for 28 days for all parameters and 90 days for HbA1c. The extraction medium contained 10 % DMSO, and the total extract volume per injection did not exceed 100 µl. In the preventive model, AME was administered first for 28 days,

followed by ALX or STZ.

2.1.2. Oral glucose tolerance test

For the oral glucose tolerance test, the normoglycemic and diabetic rats with or without AME administration were fasted overnight (16 h) and were then administered 2 g/kg dextrose solution via oral gavage. Fasting glucose and insulin levels were measured at baseline (before the glucose bolus), 30, 60, and 120 min after the glucose bolus. Blood glucose was measured using blood glucose strips (Roche Diagnostics), plasma insulin, and C-peptide.Pancreatic and hepatic glucose transporter 2 (GLUT2) were also measured using ELISA. Hepatic glucose was assayed using the glucose oxidase method. Pancreatic glutamic acid decarboxylase 2/65 (GAD2/65) and islet amyloid polypeptide (IAPP) in pancreatic homogenate were measured using ELISA. HOMA-IR was determined as follows: HOMA-IR = [glucose (mmol/L) * insulin (μU/ml)/22.5], where 1 mg/dL equals 0.055 mmol/L. The fasting β-cell responsiveness (M0) = 100 × fasting C-peptide (μg/L)/fasting glucose concentration (mg/dL).

2.1.3. Measurement of hepatic regulators of glycolysis and gluconeogenesis

Hepatic activities of hexokinase (HK), phosphofructokinase (PFK-1), pyruvate kinase (PK), phosphoenol pyruvate carboxykinase (PEPCK), and fructiose-1,6-bisphosphatase (F-1,6-BPase) were determined using their specific kits. For glucose-6-phosphatase (G-6-P) activity assay, 100 μl of liver homogenate was pipetted into a 96-well plate and incubated with 400 μl of the following reaction mixture: 0.25 M, 1 M EDTA, 0.1 M glucose-6-phosphate, 01.M cacodylate, 1.5 mM phosphate standard solution, and 1 % w/v ammonium molybdate. The reaction was allowed to stand for 30 min and stopped with 2 % ascorbic acid/trichloroacetic acid (w/v). The liberation of inorganic phosphate from G-6-P was measured at 700 nm wavelength. The concentration was expressed as μmoles/h/ml tissue protein. The enzymes were also incubated in vitro with 1–32 μg/ml of their respective inhibitors and 1–32 μg/ml AME for 2 h. Briefly, HK was incubated with 2-deoxyglucose (2-D-Glu), PFK-1 with sodium orthovanadate (Na_2V0_4), PK with shikonin, PEPCK with 3-Mercaptopropionic acid (3-MPA), F-1,6-BPase with MB05032, and G-6-Pase with dipotassium bisperoxo (picolinato) oxovanadate (V) (bpV(pic)). Glycolytic and gluconeogenic metabolites were determined as previously described[34]. Fructose-2, 6-Bisphosphate (F-2,6-BP) levels and phosphofructokinase 2 (PFK-2) activities were determined with previously described methods (Van Schaftingen and Sakata). At the endpoint, the pancreas was fixed in 10 % formalin, sectioned at 4 μm, and embedded in paraffin on glass slides. During analysis, sections were deparaffinized, rehydrated, stained with hematoxylin and eosin (H&E), and examined using a light microscopy at a magnification of X200.

2.1.4. Diet-induced model of diabetes

Rats were fed a western/ high-fat diet containing 42 % kcal from fat, 42.7 % kcal from carbohydrates, 15.2 % kcal from proteins, and 0.2 % cholesterol by weight (TD. 88137). High-fat diet (HFD) feeding lasted for 12 weeks, and AME intervention started after 8 weeks of HFD. In the intervention group, 150 mg/kg b.w PhAM was administered once every 2 weeks via oral gavage or intraperitoneal injection, while the control group received the vehicle. An oral glucose tolerance test was performed as described above.

2.1.5. Statistical analysis of the preclinical data

All statistical analyses were carried out with GraphPad Prism 10 software. All animals or data points generated were included in the study without any exclusion of outliers. Each experimental group included an n of 6 animals. Age-matched rats were randomly assigned to cages, and investigators were not blinded. All data shown were expressed as mean ± s.d. Comparison between two groups was conducted using Student's *t*-test, or one-way ANOVA among multiple groups with Tukey's test to determine statistical significance at $p < 0.05$. Significance was indicated as *.

2.2. Clinical trial

This study was carried out as a proof of concept trial to provide sufficient power for a phase 2 trial. A total of 61 participants with T2D were enrolled into the study. The study was managed by the principal investigators and clinicians who contributed to the study, and administered by the steering committee and the clinical trials office of the Imo State University. PACTR Registry approval number is PACTR202206531545729

2.2.1. Ethics statement

The safety and efficacy trials were conducted according to an approved safety management plan in compliance with the guidelines of the Imo State University ethics review board, the Association of the British Pharmaceutical Industry Guidelines for clinical trials, and the principles of the Declaration of Helsinki (2013). The trial was approved by the Institutional Ethics review board of the Imo State University with approval number IMSU/BCM/ETS/20181219. The study was also reviewed and approved by the World Health Organization International Clinical Trials Registry Platform (ICTRP), the Pan African Clinical Trial Registry, with approval number PACTR202206531545729, approved on June 15, 2022. Written informed consent or assent was obtained from all participants and no monetary compensation or inducement was provided to any of them.

2.2.2. Study design and administration, and randomization

This study was a proof of concept, single-center, double-blind, randomized, placebo-controlled trial of the safety and efficacy of PhAM in improving risk factors associated with T2D. The trial was conducted through the Department of Biochemistry and Medical Biochemistry Clinical Trial Subunit, of the Clinical trial Office of the Imo State University. The study involved patients with T2D living in 27 local governments of Imo State with a population density of approximately 4 million residents.

The fractionation of *A. mannii* plant was performed in a sterile environment at the laboratory of Medical Biochemistry, Imo State University. PhAM is wholly obtained from the phenolic acid components of *Anisopus mannii*. The protocol for extraction of PhAM is redacted, in alignment with the funders and institutional intellectual property.

The administered doses (12 and 20 mg PhAM) were estimated from the human equivalent starting dose (HESD). The HESD was estimated from the no adverse effect level (NOAEL) of PhAM observed in our preclinical trial in rats with diet-induced diabetes, as outlined by the FDA.[35]Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers Pharmacology and Toxicology Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. [35]. Based on the preclinical trial, the NOAEL was determined to be 150 mg/kg body weight (b.w) on a 200 g rat, which was equivalent to 22.8 mg/kg b.w HESD.

PhAM was administered in a blinded manner throughout the study, and participants were randomized into groups using an interactive response technology system. Participants orally received a titration of dose of 4.0 mg weekly up to 20 mg of PhAM for a month and were maintained on 12 mg or 20 mg of PhAM over 6 months. The optimum tolerable dose was 20 mg of PhAM as 25 mg caused severe diarrhea and abdominal distension. Refer to the attached study protocol for administration procedure. Placebo used was physically indistinguishable from the intervention. PhAM was administered every 48 h under the supervision of on-site nurses or compliance field officers.

2.2.3. Inclusion/exclusion criteria

Full details of the inclusion criteria is found in the attached study synopsis. The main inclusion criteria were age between ≥ 40 and ≤ 75

years, clinical diagnosis of T2D and on the spot HbA1c > 6.5 %, history of good compliance to first-line diabetes medications, and history of diabetes > 2 years. Exclusion criteria were smoking, pregnancy, breastfeeding, use of steroids or any immunosuppressive or immunomodulatory therapies, use of any herbal drugs within 2 months before trial commences, history of any other metabolic disease that complicates the pathological features of T2D, active hepatitis b or c, HIV, tuberculosis, and systemic infections.

2.2.4. Primary and secondary outcome assessments

All clinical and laboratory measurements were obtained in triplicate, and the average value was selected as representative. Baseline measurements were measured during randomization visitation, after run-in but before dosing. Height and body weight were measured at the site visit, and the average weight was used to calculate the dose of PhAM for each participant. Body mass index (BMI) was calculated as weight (kg)/(height (m))^2. As part of safety monitoring, blood pressure (Omron M6 Comfort Upper Arm Blood Pressure Monitor) and pulse rate (Handheld fingertip portable oximeter) were measured before dosing and at each dosing time. Fasted clinical laboratory tests were performed before breakfast on day 1, and weekly for the first two months, and then monthly thereafter. Venous blood samples were collected for plasma analysis of glucose (by enzymatic hexokinase method) and insulin (Human Insulin ELISA Kit, EZHI Millipore). HbA1c was measured using a Siemens/Bayer DCA 2000+ Analyzer. The primary outcome measurements for this study were the HbA1c and fasting blood glucose levels.

2.2.5. Statistical analysis

Sample size estimates were estimated to provide sufficient power for a Phase 2 trial, based on the primary outcome of the preclinical trial. According to the results of the trial, the average HbA1c of rats fed a high

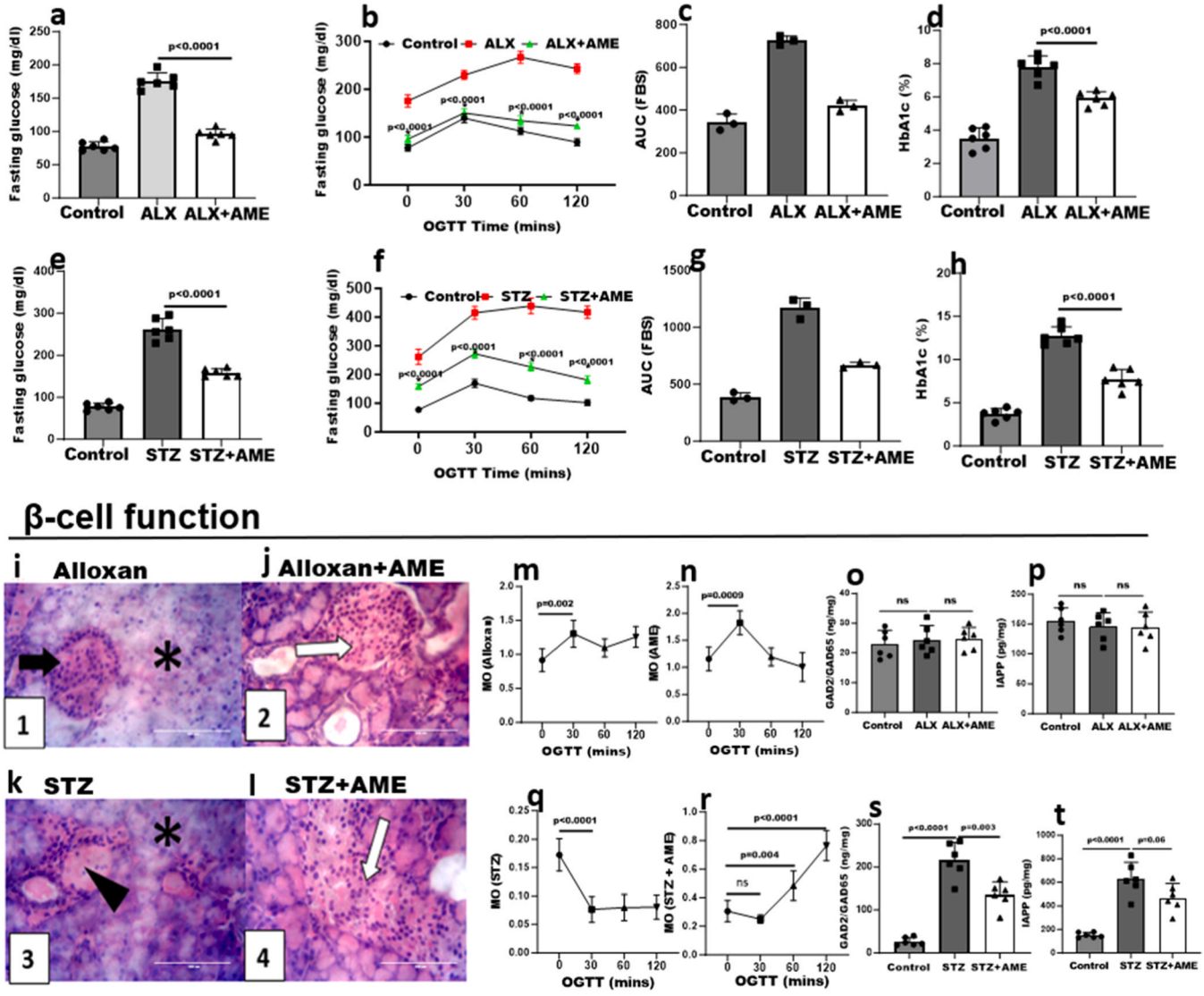

Fig. 1. *A. mannii* extract (AME) improves β-cell function in diabetic rats. n = 7. **(a,b,c,d):** Fasting blood glucose, glucose levels after oral glucose tolerance test (OGTT), area under the curve (AUC), and glycated hemoglobin (HbA1c) in alloxan (ALX)-induced diabetes, **(e,f,g,h):** Fasting blood glucose, glucose levels after oral glucose tolerance test, area under the curve (AUC), and glycated hemoglobin (HbA1c) in streptozotocin (STZ)-induced diabetes, **(i-l):** Histology of the pancreas in both ALX **(i,j)** and STZ **(k,l)**-induced diabetes, with or without AME administration. **(m,n):** β-cell responsiveness (M0) in ALX-induced diabetes (Alloxan) andALX+AME (AME), (**o,p**) Glutamic Acid Decarboxylase 2/65 (GAD2/65) and islet amyloid polypeptide (IAPP) levels in pancreatic homogenates in ALX-induced diabetes levels in pancreatic homogenates in ALX-induced diabetes, **(q,r):** β-cell responsiveness (M0) in STZ-induced diabetes and STZ+AME (AME), (**s,t**) Glutamic Acid Decarboxylase 2/65 (GAD2/65) and islet amyloid polypeptide (IAPP) levels in pancreatic homogenates in STZ-induced diabetes. Control – normoglycemic rats administered with the vehicle. ALX – Rats administered with alloxan, STZ – Rats administered with streptozotocin, AME – Anisopus mannii extract, **n = 6.**

fat diet was 7.1 %. After adding 150 mg/kg b.w PhAM, the HbA1c was reduced to 6.53 % on average, with an average difference of 0.63 %. Taking this effect size into account, the sample size was calculated using the G*Power statistical software 3.1.9.7. With a power of 90 % and one-sided $\alpha = 0.025$, 1:1:1 allocation, a total of 60 participants were required. Considering a 20 % attrition and loss-to-follow-up, 72 participants were estimated to be needed in each group. The results of the primary and secondary outcome measurements were analyzed using the Statistical Package for the Social Sciences (SPSS version 25). Data were tested for normality using Shapiro Wilk's Test. One Way ANOVA, followed by a Post-hoc test (the Fisher's Least Standard Deviation (LSD)), was applied for the statistical comparison among the placebo, 12 mg (PhAM 1), and 20 mg (PhAM 2). Graphs were made with the Graph Pad Prism.

3. Results

3.1. *A. mannii ameliorates alloxan and streptozotocin-induced dysregulation of glucose homeostasis*

We fractionated *A. mannii* into phenolic acid to isolate and identify the abundant bioactive compounds (Supplementary Fig. 1). Ferulic acid, gallic acid, protocatechuic acid, and syringic acid comprised more than 95 % of the identified phenolic compounds. We then performed viability assays on various cell lines and preclinical rat models of diabetes using trypan blue exclusion and dose-adjustment assays. We found that cells tolerated AME at < 30 μg/ml, and 100 mg/kg bw was the optimal dose in rats (Supplementary 2a and 2b). ALX and STZ were administered at 150 mg/kg bw following an 8 h fast. Except for control rats, all rats in the diabetes study were recruited after confirmation of diabetes with fasting blood glucose > 160 mg/dl. Bi-weekly administration of 100 mg/kg b.w AME to alloxan-induced diabetic rats, significantly lowered fasting blood glucose (Fig. 1a). To further buttress the glucose-lowering potential of AME, we conducted an oral glucose tolerance test in rats. At the end of the 28-day trial of AME, normoglycemic or diabetic rats with or without AME treatment received a 2 g/kg oral glucose bolus, and blood glucose was measured every 30 min until 120 min. The diabetic rats on AME treatment showed significantly improved glucose clearance at all the time points and attained near-baseline levels at 120 min (Fig. 1b and c). To discern if the improvement in glucose tolerance in AME-treated rats was independent of the low baseline glucose levels, two groups of wild type normoglycemic rats were challenged with a 2 g/kg b.w glucose bolus orally or intraperitoneally, with simultaneous oral or i.p administration of AME or vehicle. These rats had similar baseline fasting glucose levels prior to the glucose bolus challenge; however, rats concurrently treated with AME showed improved glucose clearance compared with rats treated with the vehicle (Supplementary Figure 3a, b). Adopting a preventive model, pre-administration of AME to rats bi-weekly for 6 weeks before alloxan or STZ administration significantly improved glucose tolerance (Supplementary Figure 3 e-l).

To validate the efficacy of AME in improving glycemic control in diabetic conditions, we measured the levels of HbA1c, the gold standard test for glycemic control[36]. Alloxan administration significantly elevated HbA1c levels to the diabetic range, whereas HbA1c levels in diabetic rats treated with AME were within the pre-diabetic range (Fig. 1d). Therefore, AME administration during hyperglycemia potentiates significant glycemic control. We further investigated the effect of AME on β-cell function during experimental diabetes by histological examination. Alloxan administration induced signs of severe acinar destruction and desquamation without any obvious necrosis of islet cells (Fig. 1i). The damages on acinar cells were markedly reduced following administration of AME (Fig. 1j). The pancreas of rats given STZ also showed obvious acinar destruction and desquamation, as well as severe necrosis and foci of round cell infiltration (Fig. 1k). AME administration decreased the extent of acinar damage and desquamation and abrogated round-cell infiltration foci (Fig. 1l). These findings indicate that AME protects β-cells from alloxan- and STZ-induced toxicity. We then compared the β-cell response (M0) in diabetic rats with or without AME treatment. First, we challenged these rats with 2 g/kg glucose bolus to stimulate β-cell response and measured the M0 every 30 min until 120 min. Alloxan administered rats showed increased M0 after 30 mins of glucose bolus (Fig. 1m) which remained high after 120 mins, indicating poor glucose clearance from the circulation. With AME treatment, the rats showed significantly improved M0 compared to the untreated rats after 30 and returned to baseline levels at 120 mins (Fig. 1n). To further validate that AME counteract β-cell toxicity, we measured the levels of GAD 2/65 and IAPP in pancreatic homogenates, because both are well-established biomarkers of β-cell death[37,38]. We found no significant differences in GAD2/65 and IAPP levels during alloxan-induced diabetes (Fig. 1o,p-v). This implies that alloxan administration at 150 mg/kgdid caused insignificant β-cell death. In contrast to alloxan-induced diabetes, rats administered STZ showed significantly reduced M0, which remained unchanged after a 120-min glucose challenge (Fig. 1q). This may justify the significant reduction in insulin production associated with STZ-induced type 1 diabetes (T1D). Administration of AME during in rats challanged with STZ produced no significant difference in M0 after 30 min but showed an approximately two-fold increase after 120 min of glucose challenge (Fig. 1r). This therefore emphasizes that AME administration improves β-cell response by inhibiting STZ-mediated β-cell damage. For GAD2/65 and IAPP levels in STZ-challanged rats, both GAD2/65 and IAPP levels were significantly elevated but markedly decreased after AME administration (Fig. 1s-t), demonstrating that AME counteracts STZ-induced destruction of β-cells.

3.2. *A. mannii affects hepatic glucose metabolism via phosphofructokinase agonism*

Hepatic glucose metabolism is severely dysregulated in type 1 and type 2 diabetes, and this imbalance contributes to hyperglycemia in the fasting and postprandial states[39,40]. We therefore investigated the ameliorative effect of AME on hepatic glucose homeostasis in type 1 and type 2 diabetes. Glucose transport is severely affected during diabetes, affecting hepatic glucose levels[41]. Indeed, we observed that GLUT2, the major glucose transporter of the liver, and hepatic glucose levels were severely impaired by STZ administration, but were slightly improved in rats receiving AME treatment (Fig. 2a-b). We further investigated if AME improves the metabolic breakdown of glucose by measuring the activities of hexokinase (HK), phosphofructokinase (PFK-1), and pyruvate kinase (PK), which catalyze the checkpoints of glycolysis (Fig. 2c-h). To assess if AME directly affects the enzyme activities, we incubated the enzymes with 2 – 32 μg/ml of small molecule inhibitors or AME. HK and PK activities were significantly improved in rats treated with AME during ALX and STZ-induced diabetes (Fig. 2c and g). In vitro, at all concentrations, AME showed no significant effect on HK (Fig. 2d) and PK (Fig. 2h) in the presence and absence of 2-deoxy--d-glucose (a potent HK inhibitor) or shikonin (a potent PK inhibitor) [42,43]. However, AME counteracted the inhibitory effect of sodium orthovanadate on PFK-1 activity (Fig. 2e-f). It is possible that the bioactive components of AME may modulate the activities of PFK-1 without a direct effect on hexokinase or pyruvate kinase.

We next examined the activities of enzymes regulating gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-Bisphosphatase (F-1,6-BPase), and glucose-6-phosphatase (G-6-Pase). We observed that their activities were markedly suppressed in diabetic rats treated with AME (Fig. i-n). However, AME had no direct effect on these enzymes in vitro. Therefore, AME may suppress gluconeogenesis in diabetic rats by inhibiting the activities of glycolytic enzymes indirectly. Considering that the activities of these glycolytic enzymes are regulated via substrate-level regulation or feedback inhibition, we investigated the flux of glycolytic substrates in the ALX - and STZ-induced diabetes models (Supplementary 3). Hepatic hexokinase is

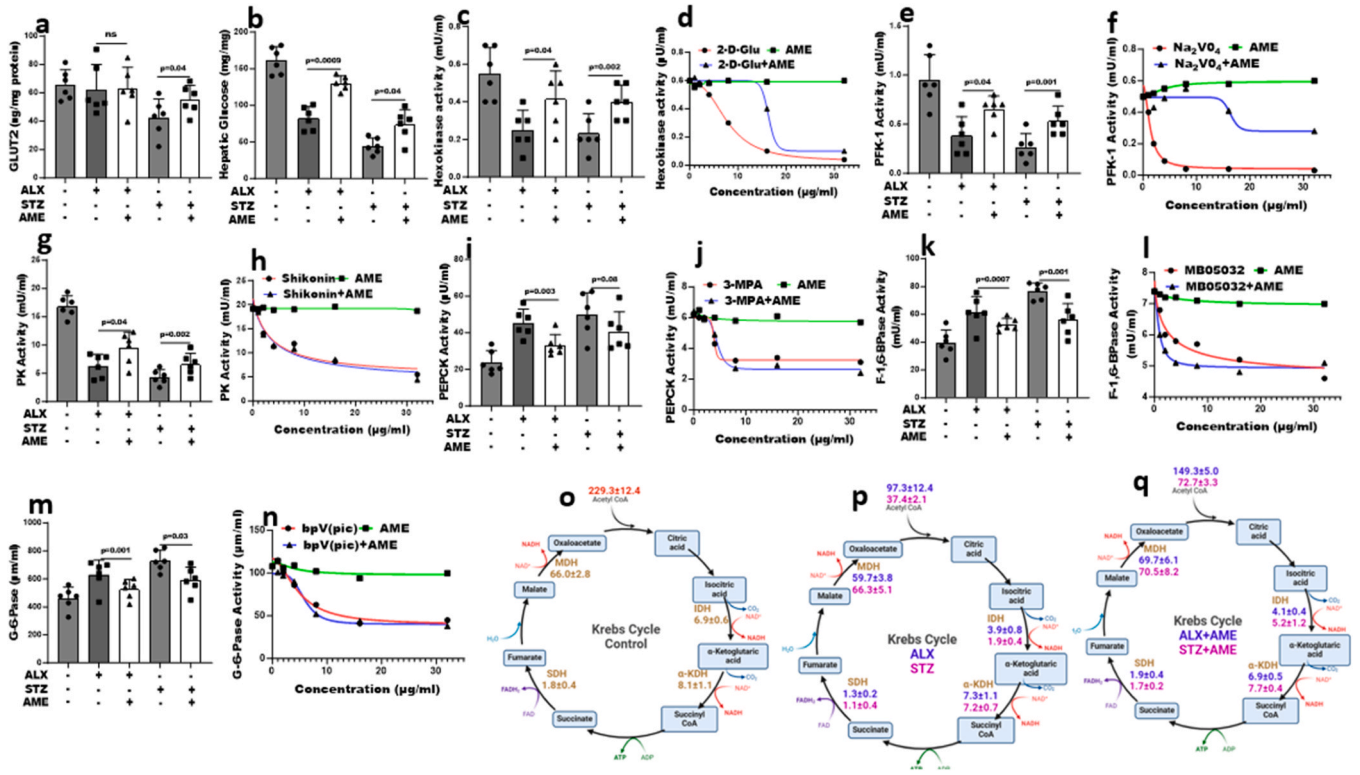

Fig. 2. *A. mannii* extract stimulates phosphofructokinase-1 activities to ameliorate hepatic glucose metabolism in type 1 and 2 diabetes. **(a)** hepatic glucose transporter 2, **(b)** hepatic glucose levels, **(c)** hexokinase activity in alloxan (ALX) and streptozotocin STZ-induced diabetes with or without AME administration, **(d)** in vitro hexokinase activity with 2-deoxy-glucose (2-D-Glu) and AME incubation, **(e)** phosphofructokinase (PFK-1) activity in ALX and STZ-induced diabetes with or without AME administration, **(f)** in vitro PFK-1 activity with sodium orthovanadate (Na_2V0_4) and AME incubation, **(g)** pyruvate kinase (PK) activity in ALX and STZ-induced diabetes with or without AME administration, **(h)** in vitro PK activity with shikonin and AME incubation, **(i)** phosphoenolpyruvate carboxykinase (PEPCK) activity in ALX and STZ-induced diabetes with or without AME administration, **(j)** in vitro PEPCK activity with 3-Mercaptopropionic acid (3-MPA) and AME incubation, **(k)** fructose-1,6-bisphosphatase (F-1,6-BPase) activity in ALX and STZ-induced diabetes with or without AME administration, **(l)** in vitro F-1,6-BPase activity with MB05032 and AME incubation **(m)** glucose-6-bisphosphatase (G-6-Pase) activity in ALX and STZ-induced diabetes with or without AME administration, **(n)** in vitro G-6-Pase activity with Dipotassium bisperoxo (picolinato) oxovanadate (V) (bpV(pic)) and AME incubation, **(o)** activities of Krebs cycle enzymes in normoglycemic rats **(p)** activities of Krebs cycle enzymes in ALX and STZ-induced diabetes, **(q)** activities Krebs cycle enzymes in diabetic rats treated with AME. The measurements in figures o, p, and q represents quantitation of the respective enzyme activities. ALX – Rats administered with alloxan, STZ – Rats administered with streptozotocin, AME – Anisopus mannii extract, **n = 6.**

activated feedforward by high glucose levels and inhibited in a feedback manner by high fructose-6-phosphate levels. GLUT2 was significantly reduced, particularly in STZ-induced diabetes, indicating that glucose transport to the liver is impaired, which may deprive the glucose-induced feedforward activation of hexokinase[44]. AME administration may improve hexokinase activity via significantly improving hepatic GLUT2 and then glucose levels.

PFK1 is regulated by the levels of its substrate and product, PEP and citrate, and is indirectly activated by F-6-P[45]. PEP, a downstream product of glycolysis, allosterically inhibits PFK1, resulting in reduced affinity of PFK1 for its substrate, F-6-P[46]. PEP levels were significantly reduced in rats treated with AME compared with untreated alloxan-induced diabetes, which may restore PFK activities in diabetic rats. In addition, we measured the flux of metabolites through the Krebs cycle pathway to understand the effect of AME administration on mitochondrial utilization of acetyl-CoA, a product of glycolysis (Fig. o-q). Hepatic acetyl-CoA levels were significantly reduced in both ALX- and STZ-induced diabetes, which were partially restored by AME treatment. Among the regulatory enzymes of the Krebs cycle pathway, only isocitrate dehydrogenase was significantly reduced in ALX- and STZ-induced diabetes compared with the control group (Fig. q vs. Fig. o), which was restored by AME treatment (Fig. P). Diabetic rats receiving AME showed a significant increase in acetyl-CoA compared with rats receiving ALX or STZ alone, which may contribute to the improvement in isocitrate dehydrogenase (IDH) levels via feedforward activation. Taken together, our results indicate that administration of AME in experimental type 1 and type 2 diabetes improves glucose sensing, transportation into hepatocytes, and glucose utilization through the glycolysis and the Krebs cycle pathways.

3.3. *Phenolic acid fractions of A. mannii modulate the activities of PFK-1 and improve glucose metabolism in high-fat diet-fed rats*

Different fractions of AME were tested in vitro to determine which fractions might modulate PFK-1. The PhAM, mainly including protocatechuic acid, gallic acid, ferulic acid, and syringic acid, markedly counteracted the inhibitory effect of sodium orthovanadate on PFK-1 (Fig. 3a). This implies that plant's phenolic fraction could be responsible for its glucose-lowering potency.

We then further tested the PhAM on a clinically relevant diet-induced model of T2D rats. The experimental rats were fed a high-fat diet and treated with or without PhAM. From the results, it was observed that PhAM induced the reduction of body weight (Fig. 3b, c). Food intake was also significantly reduced in rats receiving PhAM (Fig. 3d), which may contribute to reduced body weight. The levels of ghrelin and leptin levels (hunger hormones) were significantly lower in PhAM-treated rats, indicating decreased appetite (Fig. 3e, f). Administration of PhAM also significantly changed the appearance of the steatotic liver while no change was observed in the intestine (Fig. 3i, j). The administration of PhAM significantly reduced fasting blood glucose

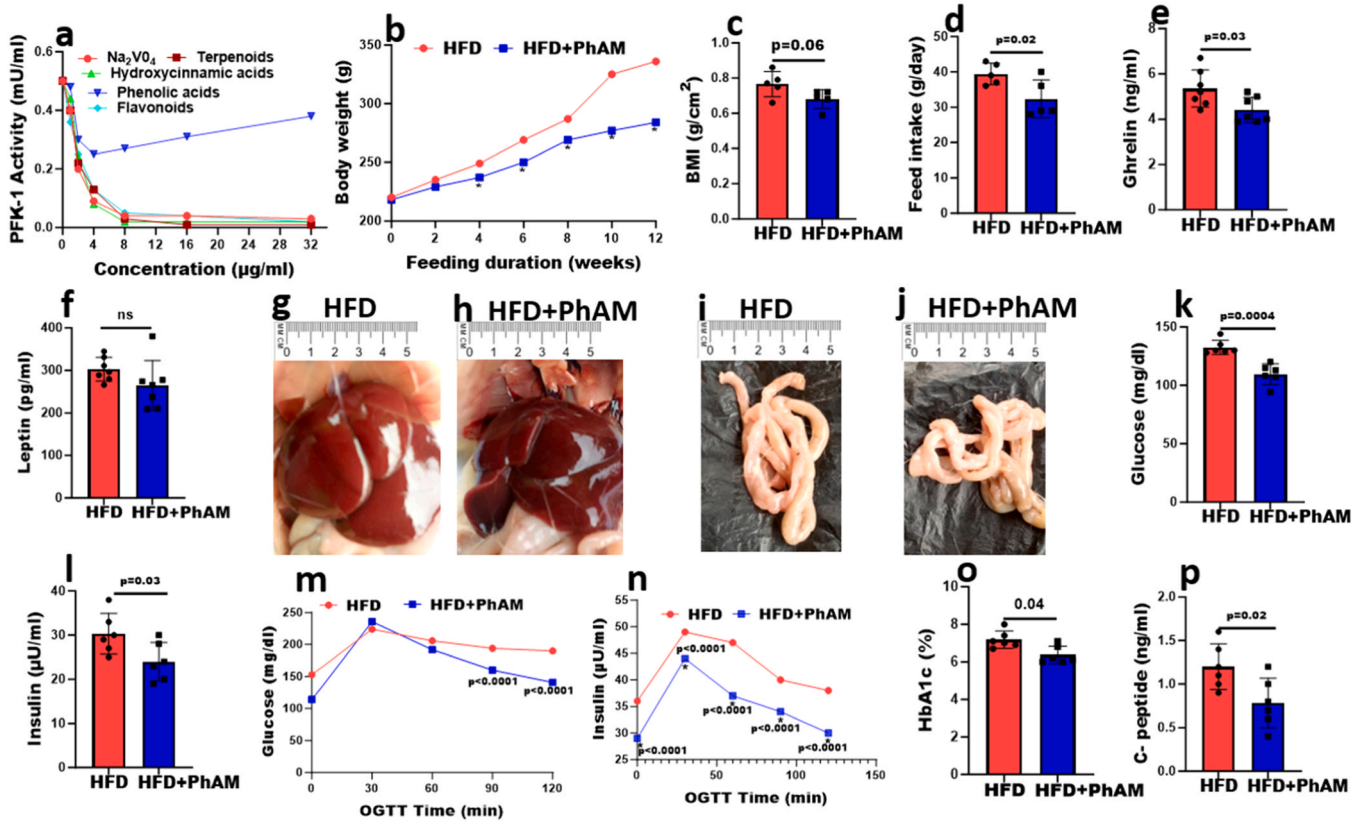

Fig. 3. *Phenolic acid extract of A. mannii (PhAM),* ameliorate dysfunctional glucose metabolism in diet-induced diabetes. **(a)** in vitro phosphofructokinase 1 (PFK-1) activities, incubated with sodium orthovanadate (Na_2V0_4) and different Anisopus mannii extract (AME) fractions **(b)** body weight, **(c)** BMI, **(d)** feed intake, **(e)** ghrelin, **(f)** leptin, levels of rats fed on high-fat diet (HFD) and treated with 150 mg/bw PhAM, **(g,h)** images of the liver of rats fed HFD with or without administration of 150 mg/bw PhAM, **(i,j)** images of the intestines of rats fed HFD with or without administration of 150 mg/bw PhAM **(k,l)** fasting blood glucose and insulin levels of rats fed HFD with or without administration of 150 mg/bw PhAM **(m,n)** glucose levels after oral glucose tolerance test (OGTT) in rats fed HFD with or without administration of 150 mg/bw PhAM **(o,p)** glycated hemoglobin (HbA1C) and C-peptide levels of rats fed high-fat diet with or without administration of 150 mg/bw PhAM. **n = 6**.

(Fig. 3k) and insulin (Fig. 3l) levels. When challenged with a glucose bolus, rats administered PhAM showed better glucose clearance at 90 and 120 min (Fig. 3m). PhAM also significantly lowered insulin levels at all time points tested (Fig. 3n). Additionally, diabetic rats treated with PhAM exhibited better glycemic control with significantly lower HbA1c and c-peptide levels compared with the control group (Fig. 3o, p). Taken together, the findings demonstrate that PhAM as a monotherapy achieves significant glycemic control in a preclinical model of diet-induced T2D.

3.4. Clinical trial population and baseline characteristics

We aimed to evaluate the clinical safety and efficacy of PhAM in patients with obesity and T2D. A total of 85 patients were screened for eligibility. 7 study participants did not show up during randomization, while 3 study participants failed elligibility (1 became pregnant, 1 had blood pressure at 175/115; meeting the criteria for screen failure, while 1 withdrew consent after not receiving assurance of being randomized into any of the PhAM group).Three participants dropped out prior to the intervention. 72 eligible participants that fulfilled the inclusion criteria were equally randomized into three groups: placebo, 12 mg PhAM (PhAM 1), and 20 mg PhAM (PhAM 2). Based on factors listed in Fig. 4, 21 (87.5 %) patients in the placebo group, 22 (91.6 %) patients in PhAM 1, and 18 (75 %) patients in PhAM 2, attained the study endpoint, which lasted for 6 months. The patients' baseline demographic and physical characteristics were listed in Table 1.

Study participants comprised of 38 %, 68 %, and 55 % females in the placebo, PhAM 1, and PhAM 2 groups, respectively. The average duration of diabetes in each group was > 7 years. Approximately 17 % of participants in the placebo group received metformin monotherapy, compared with 63 % and 72 % of metformin-only users in the PhAM 1 and 2 groups, respectively. The remaining participants took both metformin and sulphonylurea, and 1 patient had thiazolidinedione. All participants were naive of incretin, insulin therapy, or herbal medicines. The average body weight of the three study groups ranged from 94.3 to 96.7 kg, while the average BMI was approximately 33 kg/m^2. The mean HbA1c (%) levels were 7.6 in the placebo group and 7.7 in both PhAM 1 and 2 groups. HbA1c (%) levels ranged from 6.9 – 8.4 in the placebo, 6.9 – 8.5 in PhAM 1 and 6.8 – 8.9 in PhAM 2 groups (supplementary file 3). Average fasting blood glucose across the groups was between 182 – 184 mg/dl, while the mean insulin levels (µU/ml) were 18.0 in the placebo, 17.8 in the PhAM 1, and 17.2 in the PhAM 2 groups. Average HOMA-IR 1 across the study groups was between 7.7 – 8. The average blood pressure (systolic/diastolic mm/Hg) was 127.9/74.3 in the placebo group, 126.8/75.6 in PhAM 1 group, and 127/74.4 in PhAM 2 group, while the average pulse rate across the groups was between 76 – 77 bpm.

Some co-morbidities of diabetes were distributed across the study groups. Six patients in the placebo had elevated total cholesterol while eight and five study participants respectively had elevated total cholesterol levels in the 12 and 20 mg groups. In addition, 11 study participants in the placebo group had fatty liver disease while 12 in the 12 mg and 9 in the 20 mg groups had fatty liver disease respectively. None of the study participants had Polycystic ovary syndrome (PCOS), chronic kidney, lung, or thyroid disease, stroke, cancer, or retinopathy, while 2 study participants in the placebo had history of coronary artery

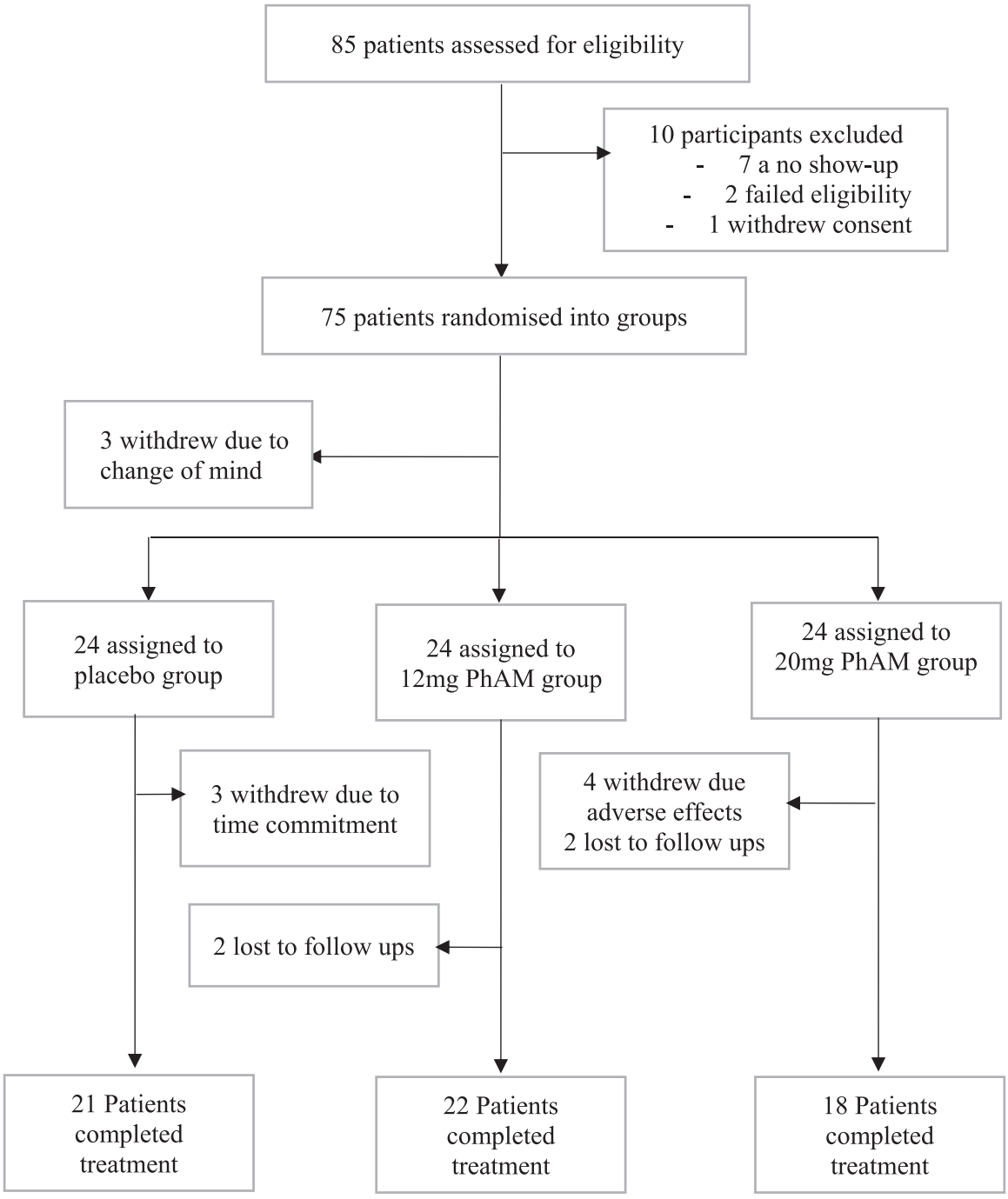

Fig. 4. Enrollment diagram (compliant with CONSORT flow diagram).

disease.

3.5. Safety and tolerability

The treatment-emergent adverse events (TEAEs) related to PhAM treatment were listed in Table 2. 12 mg of PhAM (PhAM 1) caused severe adverse effects on 13 % of participants with 9 % hospitalized, whereas 20 mg of PhAM (PhAM 2) caused severe adverse effects in 16 % of participants, 11 % of whom required hospitalization. All hospitalizations were reported to be due to severe diarrhea. No deaths or adverse effects requiring study withdrawal occurred from PhAM administration. The most reported TEAES was diarrhea and nausea, reported by 50 % of participants in PhAM 1 and 72 % of participants in PhAM 2, the majority of which occurred < 4 months without any dose adjustment. Approximately 5 % of participants in the placebo group experienced appetite loss. Therewas also an increased appetite loss (14 % and 39 % in PhAM 1 and in PhAM 2 respectively). All these effects were transient and mildly severe. Feverish condition and sleepiness reported by participants did not appear to be related to the study, as the majority occurred in the placebo group. 27 % of participants in PhAM 1 and 33 % of participants in PhAM 2 reported occasional tiredness, while one participant in the PhAM 2 group showed tiredness with occasional vomiting. Two patients in PhAM 1 or 2 experienced constipation and abdominal distension, while 4 patients reported flatulence and eructation. None of these participants had dyspepsia, palpitations, or hypoglycemia.

Table 1
Baseline demographic characteristics of study participants.

	Placebo (n = 21)	12 mg PhAM (n = 22)	20 mg PhAM (n = 18)
Sex (%)			
Male	13 (62)	7 (32)	8 (45)
Female	8 (38)	15 (68)	10 (55)
Average Age in years (mean ± SD)*	52.9 (3.7)	50.8 (5.6)	52.0 (5.1)
Average duration of diabetes in years (mean ± SD)*	7.5 (2.1)	8.6 (1.5)	7.9 (1.9)
Drugs			
Metformin	17 (80.9)	14 (63.6)	13 (72.2)
Metformin + Sulphonylureas	3 (14.2)	8 (36.3)	5 (27.7)
Thiazolidinedione	1 (4.7)	0	0
Incretins	0	0	0
Insulin therapy	0	0	0
Herbal drugs	0	0	0
Body weight (g)*	94.3 (11.7)	96.7 (9.7)	94.7 (11.2)
Height (cm)*	166.2 (10.3)	170 (10.7)	167.6 (12.1)
BMI (kg/m^2)*	33.8 (2.2)	33.3 (2.1)	33.5 (1.8)
HbA1c (%)*	7.6 (0.4)	7.7 (0.5)	7.7 (0.5)
Glucose (mg/dl)*	183 (20.6)	184 (18.8)	182 (23.9)
Insulin (μU/ml)*	18.0 (2.1)	17.8 (2.0)	17.2 (2.4)
HOMA-IR*	8.1 (1.1)	8.0 (1.4)	7.7 (1.7)
Blood pressure (mm/Hg)			
Systolic*	127.9 (4.9)	126.8 (7.0)	127.0 (8.0)
Diastolic*	74.3 (4.0)	75.6 (3.3)	74.4 (4.1)
Pulse rate (bpm)*	77.7 (9.1)	77.6 (9.2)	76.2 (8.3)

Data are n (%) or mean. HOMA-IR = (glucose x insulin)/405. BMI, body mass index. HbA1c – glycated hemoglobin. For drugs and sex, data are presented as counts (%), while every other data are presented as mean (SD). * - no significance changes in statistical comparison among the three groups

Table 2
Safety profile of study participants.

Safety profile	Placebo (n = 21)	12 mg PhAM (n = 22)	20 mg PhAM (n = 18)
Severe adverse effects	0	3 (13.6)	3 (16.6)
Hospitalization	1 (4.7)	2 (9.0)	2 (11.1)
Deaths	0	0	0
After effects leading to withdrawal	0	0	0
Persistent Diarrhea	0	11 (50)	13 (72.2)
Diarrhea > 4 months	0	3 (13.6)	6 (33.3)
Appetite loss or decrease	1 (4.7)	5 (22.7)	7 (38.8)
Nausea	2 (9.5)	12 (54.5)	13 (72.2)
Cough	0	0	0
Fever	5 (23.8)	3 (13.6)	1 (5.5)
Muscle pain	5 (23.8)	0	0
Back pain	0	0	0
Sleepiness	3 (14.2)	0	0
Tiredness/weakness	1 (4.7)	6 (27.2)	6 (33.3)
Vomiting	0	0	1 (5.5)
Constipation	0	2 (9.0)	2 (11.1)
Abdominal distension	1 (4.7)	2 (9.0)	2 (11.1)
Flatulence	0	2 (9.0)	4 (22.2)
Eructation	0	2 (9.0)	4 (22.2)
Dizziness	0	0	0
Rash	2 (9.5)	0	0
Dyspepsia	0	0	0
Palpitations	0	0	0
Hypoglycemia	0	0	0

Data are n (%)

3.6. Primary endpoint

One Way ANOVA analysis revealed that patients had a comparable baseline HbA1c in the three groups (Fig. 5a). After three months of treatment (midpoint), 12 mg of PhAM produced no significant effect on patients, but 20 mg PhAM achieved a mild but statistically significant reduction (Fig. 5b). At the endpoint (6 months after PhAM administration), patients treated with 12 mg or 20 mg PhAM recorded a significant reduction HbA1c (Fig. 5c). Also, fasting blood glucose levels were comparable among the three groups at baseline (Fig. 5d), whereas only 20 mg of PhAM significantly reduced fasting glucose levels three months after the intervention (Fig. 5e). At 6 months of intervention, both 12 mg of PhAM and 20 mg of PhAM significantly reduced fasting blood glucose levels (Fig. 5f).

To further disaggregate the results of the primary endpoints, HbA1c and fasting blood levels of each patient were compared at baseline, three months after intervention (midpoint), and 6 months after intervention (the endpoint), as presented in Table 3. Three months after treatment, fewer patients reported further increase in HbA1c from baseline after taking 12 mg or 20 mg of PhAM than in the placebo group. Approximately 28.6 % of participants in the placebo group reported higher HbA1c after 3 months, whereas only 4.5 % of patients receiving 12 mg of PhAM showed a further increase in HbA1c from baseline, and all patients receiving 20 mg of PhAM showed an improvement in HbA1c compared with baseline. These findings indicate that combining PhAM with existing antidiabetic drugs controls any further increase in HbA1c levels. The result also showed that HbA1c levels improved in most participants who received 12 mg or 20 mg of PhAM. After three months, approximately 19 % of participants in the placebo group recorded no change in their HbA1c levels, compared with only 9 % of participants in the PhAM1 group. 63.7 % and 22.7 % of participants in the PhAM1 group experienced a reduction in HbA1c of 0.1–0.4 and 0.5–1.0, respectively. Furthermore, 83.3 % of patients in the PhAM2 group achieved a 0.5–1.0 reduction in HbA1c. After 6 months of treatment, all patients who received 12 mg or 20 mg of PhAM showed measurable progress in their HbA1c levels. After taking 12 mg of PhAM, 45.5 % of participants experienced a 0.5 – 1.0 % reduction in HbA1c, while 77.8 % of participants taking 20 mg of PhAM experienced a 1.1 – 2.0 % reduction in HbA1c. Furthermore, only 9 % of participants who received 12 mg of PhAM reported further increases in fasting blood glucose, while all patients who received 20 mg of PhAM had improvements in fasting blood glucose. In addition, only patients (13.6 %) in PhAM 1 and (55.6 %) in PhAM 2 showed a decrease in fasting blood glucose to between 21 and 40 mg/dl, whereas a reduction between 41 – 70 mg/dl only occurred in participants who received 20 mg of PhAM (22.2 %). At the endpoint, 33 % of patients in the placebo group recorded fasting blood glucose levels higher than their respective baselines. 50 % of participants who received 12 mg of PhAM recorded a decrease in fasting blood glucose by 21 – 40 mg/dl, whereas 50 %, 38.9 %, and 11.1 % of participants in the PhAM2 group experienced a reduction in fasting blood glucose of 21–40 mg/dl, 41 – 70 mg/dl, and > 70 mg/dl, respectively.

3.7. Secondary endpoint

We further compared patients' insulin and HOMA-IR levels and BMI in the placebo group with that in the PhAM1 and PhAM2 groups at baseline and at 3 and 6 months of treatment (Fig. 5). Baseline insulin levels were comparable among the three groups (Fig. 6a). At the endpoint, patients who were administered 12 mg of PhAM showed significantly lower insulin levels compared with the placebo group. The insulin levels were further reduced in patients taking 20 mg of PhAM (Fig. 6b). No significant difference was observed in baseline HOMA-IR levels between the placebo group and the intervention groups (Fig. 6c). However, at the endpoint, HOMA-IR levels were significantly lower in patients who received 12 mg or 20 mg of PhAM than in the placebo (Fig. 6d). In addition, the baseline BMI of the intervention groups was comparable to that of the placebo group (Fig. 6e). However, after 6 months of treatment, the BMI of patients taking 12 mg or 20 mg of PhAM were significantly lower than that in the placebo group (Fig. 6f).

We also stratified the secondary outcome measurements into

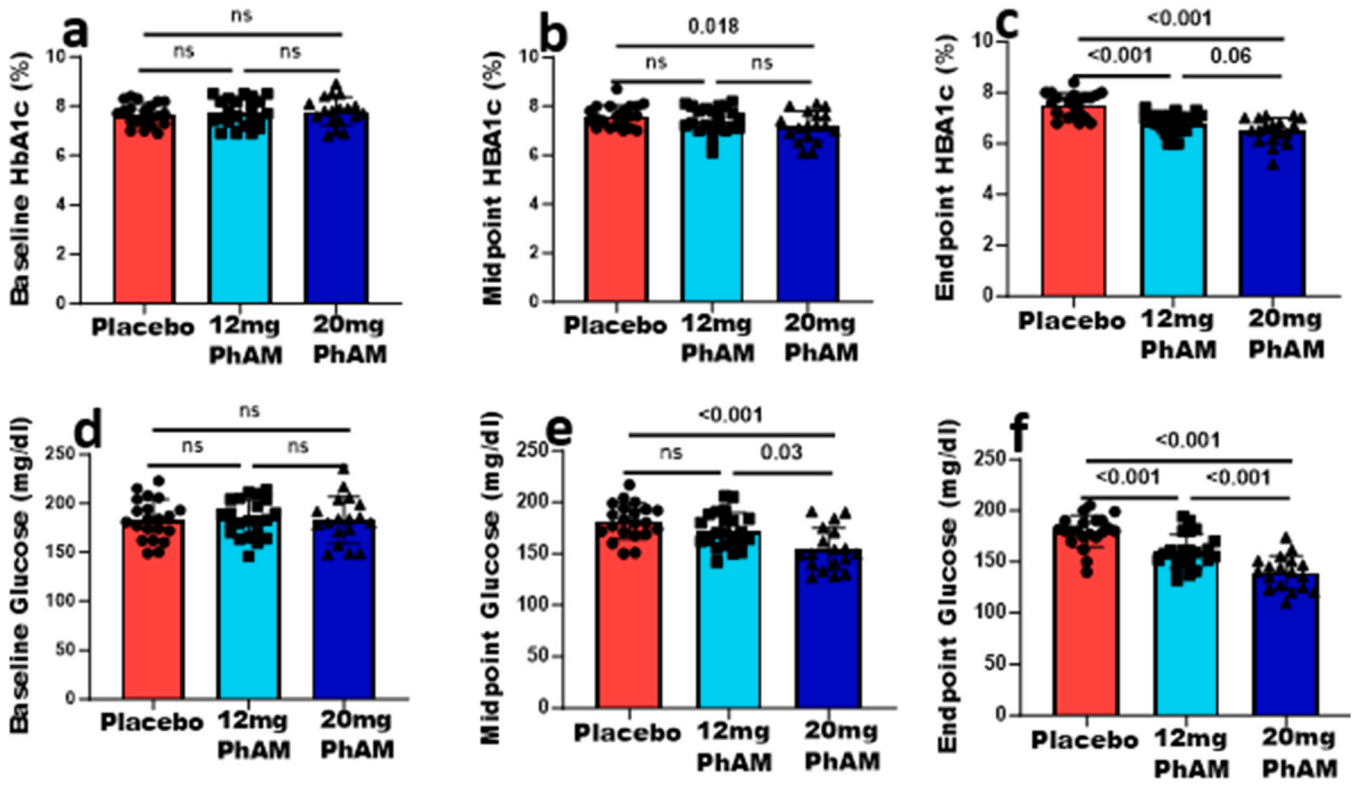

Fig. 5. Primary endpoint measurements of diabetic patients administered placebo, 12 mg or 20 mg of Phenolic acid fractions of *Anisopus manni (*PhAM). **a)** baseline glycated hemoglobin (HbA1c), **b)** midpoint HbA1c, **c)** endpoint HbA1c, **d)** baseline fasting blood glucose, **e)** midpoint fasting blood glucose, **f)** endpoint fasting blood sugar.

Table 3
Disaggregation of primary outcomes.

	Midpoint			Endpoint		
Number of patients (%)	Placebo	PhAM 1	PhAM 2	Placebo	PhAM 1	PhAM 2
Changes in HbA1c						
Worsened	6 (28.6)	1 (4.5)	0	6 (28.6)	0	0
No change	4 (19.0)	2 (9.0)	0	1 (4.8)	0	0
Decrease between 0.1 and 0.4	9 (42.9)	14 (63.7)	2 (11.1)	8 (38.0)	2 (9.0)	0
Decrease between 0.5 – 1.0	2 (9.5)	5 (22.7)	15 (83.3)	6 (28.6)	10 (45.5)	4 (22.2)
Decrease between 1.1 – 2.0	0	0	1 (5.6)	0	10 (45.5)	14 (77.8)
Changes Fasting Blood Glucose						
Worsened	6 (28.6)	2 (9.1)	0	7 (33.3)	0	0
Decrease < 10 mg/dl	8 (38.1)	8 (36.4)	2 (11.1)	4 (19.0)	6 (27.3)	0
Decrease between 11 – 20 mg/dl	7 (33.3)	9 (40.9)	2 (11.1)	6 (28.6)	2 (19.1)	0
Decrease between 21 – 40 mg/dl	0	3 (13.6)	10 (55.6)	4 (19.0)	11 (50)	9 (50)
Decrease between 41 – 70 mg/dl	0	0	4 (22.2)	0	3 (13.6)	7 (38.9)
Decrease > 70 mg/dl	0	0	0	0	0	2 (11.1)

different levels of changes observed in participants (Table 4). Fewer participants reported a further increase in insulin levels after treatment with 12 mg of PhAM, compared with that in the placebo group (4.6 % vs. 23.8 %). Many patients (36.4 %) who received 12 mg PhAM showed a decrease in insulin levels by 1.1 – 2.0 μu/ml. Only patients who received 12 mg or 20 mg of PhAM showed a decrease in insulin levels by 4.1 – 5.0 or > 5.0, respectively. Approximately 13.6 % and 9.1 % of participants taking 12 mg of PhAM had insulin levels reduced by 4.1–5.0 and > 5.0, respectively. Treatment with 20 mg of PhAM reduced insulin levels by 4.1 – 5.0 and > 5.0 in 33.3 % and 44.4 % of patients, respectively. Furthermore, HOMA-IR levels improved in all patients who received 12 mg or 20 mg of PhAM. Among the patients in the PhAM 1 group, 45.5 % recorded a decrease in HOMA-IR by 1.1 – 2.0 levels, while 22.7 % showed a decrease by 2.1 – 3.0 levels. Of patients who received 20 mg of PhAM, 33.3 % showed a decrease in HOMA-IR between the 2.1 – 3.0 level. Compared with the placebo group, only patients taking 12 or 20 mg of PhAM had a reduction in HOMA-IR between the 3.1 – 4.0 and the 4.1 – 5.0 level, respectively. Regarding changes in BMI, 27.3 % of patients taking 12 mg of PhAM experienced a 1.1 – 2.0 decrease. Half of patients (50 %) who received 20 mg of PhAM showed a decrease in BMI between 1.1 – 2.0. The administration of 20 mg of PhAM reduced BMI to > 1.0 in all patients, with 33.4 % and 38.9 % of patients achieving a reduction in BMI to a level of 2.1 – 3.0 and 3.1 – 4.0, respectively.

4. Discussion

The preclinical trial of *A. mannii* led to the fractionation and identification of PhAM as the potential bioactive compounds with anti-hyperglycemic effects. We also performed a double-blind RCT in patients with T2D to evaluate whether PhAM is better than placebo as an

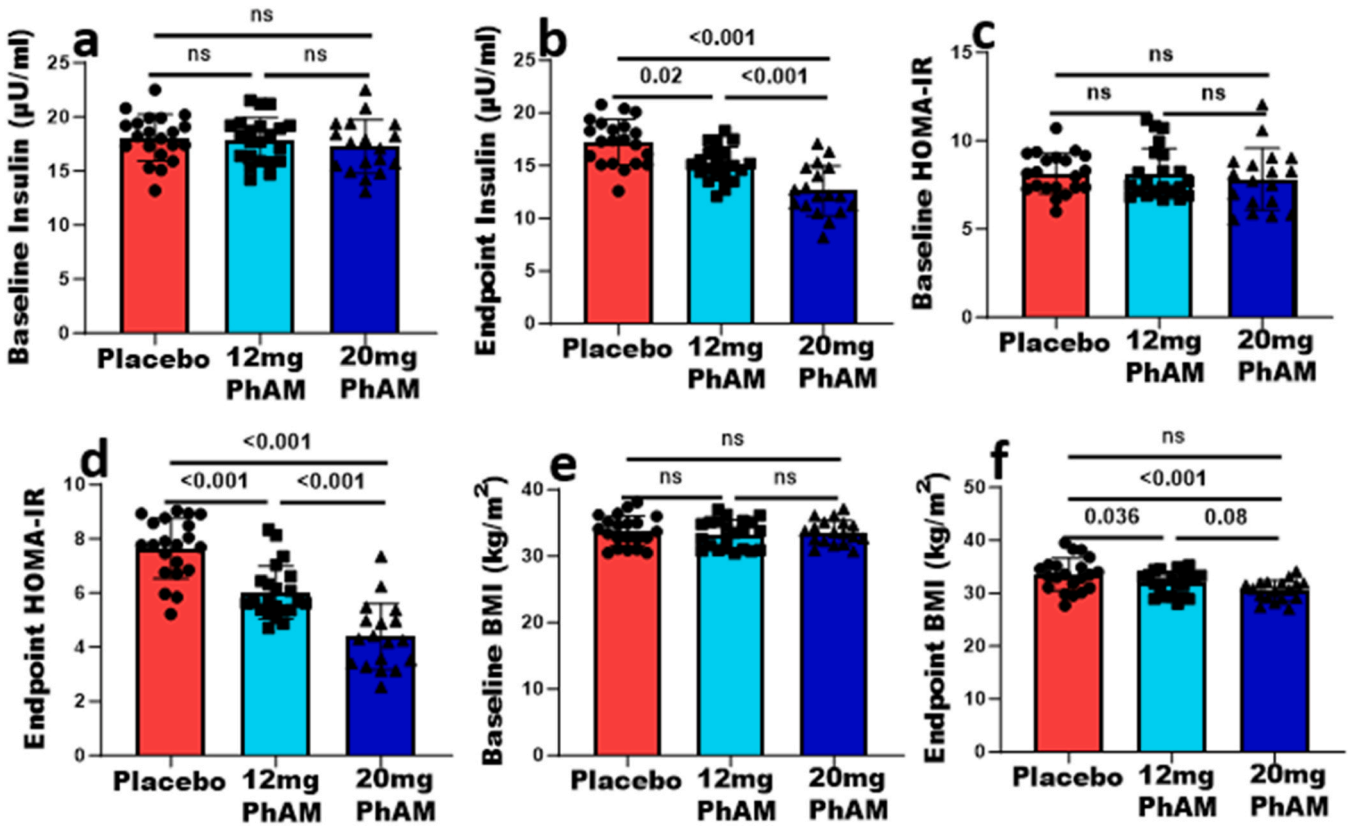

Fig. 6. Secondary endpoint measurements of diabetic patients administered placebo, 12 mg or 20 mg of Phenolic acid fractions of *Anisopus manni (*PhAM). **a)** baseline glycated hemoglobin (HbA1c), **b)** midpoint HbA1c, **c)** endpoint HbA1c, **d)** baseline fasting.

Table 4
Disaggregation of secondary outcomes.

	Endpoint		
Number of patients (%)	Placebo	PhAM 1	PhAM 2
Changes in Insulin Levels			
Worsened	5 (23.8)	1 (4.6)	0
Decrease between 0.5 – 1.0	5 (23.8)	2 (9.1)	2 (11.1)
Decrease between 1.1 – 2.0	4 (19.0)	8 (36.4)	1 (5.6)
Decrease between 2.1 – 3.0	4 (19.0)	3 (13.6)	1 (5.6)
Decrease between 3.1 – 4.0	3 (14.2)	3 (13.6)	0
Decrease between 4.1 – 5.0	0	3 (13.6)	6 (33.3)
Decrease > 5.0	0	2 (9.1)	8 (44.4)
Changes in HOMA-IR			
Worsened	7 (33.3)	0	0
Decrease between 0.5 – 1.0	6 (28.5)	3 (13.6)	0
Decrease between 1.1 – 2.0	5 (23.8)	10 (45.5)	1 (5.6)
Decrease between 2.1 – 3.0	4 (19.0)	5 (22.7)	6 (33.3)
Decrease between 3.1 – 4.0	0	2 (9.1)	5 (27.7)
Decrease between 4.1 – 5.0	0	2 (9.1)	5 (27.7)
Decrease > 5.0	0	0	1 (5.5)
Changes in BMI			
Worsened	8 (38.1)	1 (4.5)	0
Decrease between 0.5 – 1.0	5 (23.8)	6 (27.3)	0
Decrease between 1.1 – 2.0	6 (28.6)	11 (50)	3 (16.7)
Decrease between 2.1 – 3.0	2 (9.5)	3 (13.6)	6 (33.4)
Decrease between 3.1 – 4.0	0	1 (4.5)	7 (38.9)
Decrease between 4.1 – 5.0	0	0	1 (5.5)
Decrease > 5.0	0	0	1 (5.5)

antidiabetic agent in modulating circulating markers relevant to the pathophysiology of T2D. *A. mannii* has been traditionally used since ancient times to successfully manage hyperglycemia[17-19]. However, to date, no studies have investigated the therapeutic effects of its bioactive compounds. We demonstrate that the herb, especially PhAM, modulates the activities of PFK-1, an allosteric enzyme that catalyzes the rate-limiting step of glycolysis. In addition, PhAM protected pancreatic beta cells from further damage induced by diabetogenic compounds, such as ALX and STZ. Diabetes dampens cellular glucose utilization through dysregulation of enzymes participating in glycolysis and the Krebs cycle, which may starve essential metabolites needed for normal glucose homeostasis[39,40].

Our results provide valuable insights into utilizing bioactive components of *A. mannii* to target key pathways dysregulated in T2D. Of interest is the potential of AME to rescue phosphofructokinase from sodium orthovanadate-induced inhibition. Given this effect, our study provided important evidence for the identification of compounds relevant to the druggability of PFK-1. PhAM rich in ferulic acid, gallic acid, protocatechuic acid, and syringic acid, showed strong modulation of PFK-1 activity. In addition, PhAM reduced insulin, fasting blood glucose, and body weight, and enhanced glucose clearance when diabetic rats received a glucose bolus. These findings indicate a promising preclinical outcome with significant translational potentials because administration of PhAM maintains a target level of HbA1c and fasting blood sugar without compromising safety and tolerability.

PhAM, just like the whole extract the AME, improved the activities of PFK-1 in the preclinical trial. All other glycolytic enzyme activities were significantly improved in the diabetic mice that received AME, but only PFK-1 activities improved in vitro with either AME or PhAM incubation. This therefore demonstrated the specificity of PFK-1 for PhAM. PFK-1 is the rate limiting enzyme of the glycolytic pathway, the main pathway for cellular glucose utilization. PFK-1 catalyzes the phosphorylation of fructose 6 phospate to fructose 1,6-bisphosphate. It is well established that during diabetes, phosphofructokinase activities are impaired as well as other enzymes of the glycolytic pathway, which agrees with the present findings of this study[47,48]. The suppression of PFK-1 during diabetes relates majorly to the decreased levels of fructose-2, 6-phosphate, the most potent agonist of PFK-1 regulated by insulin

signalling[49,50]. To succintly put, the impairment in insulin signalling during diabetes impairs the production of fructose-2,6-bisphosphate which in turn diminishes the activities of PFK-1. Fructose-2,6-bisphosphate is produced by a close family member of PFK-1 called the PFK-2 [51]. Thus, we also explored the potentials that PhAM many have improved the activities of PFK-1 by increasing the production of fructose-2,6-bisphosphate possibly through the increase in PFK-2 activities. However, both the levels of F-2,6-BP and activities of PFK-2 remained unaffected by the PhAM administration (as shown in the supplementary figure 4). Since glycolysis and gluconeogenesis dynamically regulate each other, the impairment in PFK-1 which impairs glycolysis, elevates gluconeogenesis and perpetuates hyperglycemia. This may explain the significant decrease in the activities of the enzymes of gluconeogenesis following the administration of AME. Thus, the findings of our preclinical trial shows that PhAM produces a promising effect on glycosis by directly improving the activities of PFK-1 which improved glycemic control during experimental diabetes.

Furthermore, as our findings revealed, PhAM are mostly comprised of phenolic acids that are well documented to produce antioxidants and lipid lowering effects. Both antioxidant and anti-hyperlipidemic therapies have shown promising effects in various experimental and clinical models of diabetes. Ferulic acid, one of the phenolic acids in PhAM is among the most widely researched phenolic acid, well documented for both its antioxidant and lipid lowering potentials. Ferulic acid reportedly decreased triglycerides and total cholesterol in a randomized, double-blind, placebo-controlled clinical trial[52]. In addition to this, and in agreement with the findings of this study with the AME, ferulic acid was reported to inhibit the activities of PEPCK and G-6-Pase which diminished hepatic glucose production and hyperglycemia[53]. The antidiabetic effects of gallic and procatechuic acids which were other abundant phenolic acid in the PhAM, have been linked to their antioxidant effects[54]. Gallic acid reportedly prevents DNA oxidative damage in T2D patients (Ferk), and when combined with metformin, improved glucose metabolism and antioxidant status in diabetic rats[55]. Similarly, protocathechuic acid has been shown to produce hepatoprotective effects during T2D by ameliorating lipid accumulation, oxidative stress, and through the NF-κB and Wnt1/β-catenin pathways[56]. In addition, Wang et al., revealed that gallic acid improved diabetic steatohepatitis in experimental animals, by alleviating hepatic inflammation and lipid accumulation, while improving the activities of antioxidant enzymes [57]. In a similar fashion, syringic acid ameliorated STZ-induced chronic hyperglycemia through its potentials to ameliorate mitochondrial biogenesis and oxidative stress[58]. Thus, these previous reports on the abundant phytochemical components of the PhAM indicate the possibility that other mechanisms such as antilipidemic and antioxidant properties may contribute to the effect of PhAM on glycemic control.

In a double-blind RCT, PhAM taken with first-line anti-hyperglycemic medications for 6 months resulted in significant improvements in HbA1c and fasting glucose levels compared with placebo. Administration of PhAM was shown to be well tolerated and generally safe over a 6-month administration. Common adverse effects observed were diarrhea which resolved with continued use in most patients, appetite loss, nausea, and tiredness, but none of these is life-threatening. Patients' vitals, particularly their blood pressure and pulse rate, were essentially comparable between the placebo and treatment groups and remained unchanged during PhAM administration. The safety and tolerability profile of PhAM appear to be similar to other classes of popular drugs used to treat diabetes, especially considering that, like PhAM, other drugs, such as metformin, pioglitazone, glyburide, voglibose, and repaglinide, have been well documented to cause diarrhea and nausea [59-63]. Interestingly, many of the adverse effects reported in the placebo group, such as fever, muscle pain, sleepiness, and rash, occurred in few or no patients treated with PhAM.

In addition, all patients receiving 20 mg of PhAM, despite the side effects, showed major improvement in HbA1c as early as 3 months, with the majority of patients achieving a reduction in HbA1c by 0.5 – 1.0. A key finding was that PhAM 1 and PhAM 2 reduced HbA1c levels by 0.5 – 2.0 and fasting blood glucose by > 20 mg/dl in most patients at the endpoint. Considering that these patients are not naive to diabetes treatment, the robust reduction in HbA1c and fasting glucose levels achieved by PhAM represents a promising outcome for the clinical management of diabetes. In other studies, metformin in combination with other oral anti-hyperglycemic treatments or insulin for 6 months reduced HbA1c by 0.95 and 0.83 %, respectively, in patients with T2D [64]. Another study also showed that metformin (2500 mg/day) combined with rosiglitazone (4 mg/day or 8 mg/day) for 6 months reduced HbA1c by 1–1.2 %. The additional glycemic control achieved by the co-administration of a first-line medication and PhAM may be due to the synergistic effects of different components in PhAM. Notably, 66 % of patients receiving 20 mg of PhAM met the American Diabetes Association-recommended HbA1c goal of < 7 %, with the remaining 33 % of patients having HbA1c levels between 7 – 7.1 %[65]. This efficacy achieved by PhAM appears to be similar to that achieved by the combination of sitagliptin, a dipeptidyl peptidase-4 inhibitor, and metformin[66]. It is well established that the best efficacy of most anti-hyperglycemic agents is seen in patients with very high baseline HbA1c. However, after 6 months of taking PhAM, 90 % of patients with baseline HbA1c between 6.8 – 7.9 % achieved HbA1c in a prediabetes range of < 6.5 %. This is remarkable given that the use of popular antidiabetic drugs in patients with HbA1c < 7 % often fails to achieve sufficient glycemic control[67].

Furthermore, the results of secondary endpoints suggest that both 12 mg and 20 mg of PhAM improved insulin sensitivity in patients with diabetes. All patients in this study had baseline HOMA-IR levels > 4.0, indicating the presence of IR, whichworsened in 33 % of patients despite taking first-line medications. These findings are consistent with those from two other major studies (UK Prospective Diabetes Study [UKPDS] and A Diabetes Outcome Progression Trial [ADOPT]) showing that despite intervention, IR worsens, driving glycemic progression[68,69]. However, we observed that administration of PhAM curtailed further worsening of IR and achieved a reduction of > 1.0 in over 80 % of participants. Most first-line monotherapies or combination therapies for diabetes have been reported to be ineffective in reducing HOMA-IR after 6 months or achieving an IR reduction by < 1.0[70]. Notably, combination of 20 mg of PhAM with other first-line diabetes treatment led to a reduction of IR > 4.0 in approximately 60 % of participants after 6 months of treatment. Only a few combination therapies (e.g. semiglutide + metformin) have been reported to result in > 3.0 reduction in IR, primarily due to extensive weight loss. Like these therapies, PhAM administration reduced BMI by > 1.0 in more than 60 % of participants in the intervention group, which was expected given that most participants on PhAM reported persistent diarrhea and nausea for more than 3 months after study commencement. Similar observations have been reported elsewhere with traditional weight loss therapies, such as orlistat and cetilistat, which cause diarrhea and nausea, indirectly improving BMI and IR[71,72]. Thus, the reduction in BMI achieved with PhAM, despite causing persistent diarrhea, may contribute to ameliorating pathologic deficits associated with T2D.

One of the major strengths of this study is demonstration of the translational significance of a popular herb used in Nigeria for the treatment of diabetes, which is highly prevalent in Nigeria. By identifying and fractionating the antidiabetic compounds in this herb, the study successfully circumvented the dosage, reproducibility, and potentially life-threatening side effects associated with the use of the herbal therapy, which have hindered its incorporation into orthodox use. Another novel aspect of this study is the exploitation of the druggability of PFK-1, which may improve the clinical sequelae of T2D compared with conventional treatments. Therefore, by identifying PhAM as an actual antidiabetic compound that improves clinical outcomes in T2D, the results of this study are highly generalizable to clinical practice in Nigeria and worldwide as well.

In summary, the preclinical evidence from this study suggests that

PhAM modulate PFK-1 activity and protect β-cells from destruction, which ameliorates HFD-induced hyperglycemia while 12 mg and 20 mg of PhAM and improved the clinical sequelae of T2D, including the levels of HbA1c and fasting blood glucose, insulin sensitivity, and body mass index.

Limitations

The study also had several limitations, including a relatively small number of participants, and a lack of control of the administration of the first-line treatment of diabetes among the study participants. Also, an unequal sex distribution across the groups of the participants is a potential confounding factor to the efficacy of these interventional products. The type of firstline anti-diabetic drug taken by these participants also varied among the study groups, which could confound the efficacy of the investigational products administered. Another limitation was the lack of extensive titration of PhAM to adequately separate any dose-dependent effect of PhAM. In addition, the influence of diet or physical activity cannot be controlled, which directly affects the efficacy of the medications used for the conventional management of T2D.

Future directions

This study has provided the results required to carry out a sufficiently powered phase 2 trial with the aim of progressing to a Phase III trial. PhAM provided superior glycemic control compared to the placebo. With these, the future direction would entail the clinical trial of the PhAM in a larger sample size in an equally distributed male and female participants. In addition to this, PhAM improved beta cells function after streptozotocin administration, a feature that could benefit patients with type 1 diabetes. Hence, the clinical trial of PhAM on patients with type 1 diabetes, could be considered in future studies. Also, the clear amelioration of the fatty liver phenotype should instigate future studies to consider the clinical efficacy of PhAM in patients with fatty liver and metabolic-dysfunction associated steatohepatitis. It will also seem to be a promising future direction for studies to compare the effects of the individual phenolic acids to a synergistic effect of the entire phenolic acids fraction.

CRediT authorship contribution statement

Chiamaka W. Adumekwe: Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Ailun Gaowa:** Validation, Methodology, Investigation, Data curation. **Oluchi Aloy-Amadi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation. **Govind S. Gill:** Writing – review & editing, Validation, Methodology, Data curation. **Emmanuel N. Agomuo:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chidi N. Ekweogu:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis. **Suha J. Jarad:** Writing – review & editing, Visualization, Investigation. **Dawei Zhang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Peter Uchenna Amadi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Barbora de Courten:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization. **Justice O. Osuoha:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Chioma Ejiofor:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Esienanwan E. Efiong:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Data curation. **Prince C. Odika:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are very grateful to the GPs affiliated with Imo State University for assisting in patient recruitment and management and overseeing the administration of PhAM. We are indeed grateful to the support of Imo State University clinical trial management committee of the Faculty of Biological Sciences, and the expert contributions of the staff of the Faculty of Medicine, Imo State University. Our gratitude also goes to the department of plant science and biotechnology for the consistent support in growing the herb, and to all the community heads and field workers, disease surveillance team, and every other field worker that contributed to the success of this study.

This study was funded from grants awarded to Dr. Peter Amadi by Tetfund; TETF/DASTD/UNIV/ OWERRI/TSAS/2020 /VOL.1 and University of Cambridge and Alborada Foundation: G115009. Tetfund Grant Award to Dr. Justice Obinna Osuoha: TETF/ES/POLY/IMO STATE/TSAS/2019, and TETF/DESS/POLY/OMUMA/IBR/2023/VOL.I and Canadian Institute of Health Research Project Grant Award to Dr. Dawei Zhang: CIHR-IRSC: PS 178091.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2025.107602.

Data availability

Data will be made available on request.

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