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ORIGINAL ARTICLE

Amelioration of hyperglycemia and hyperlipidemia in a high-fat diet-fed mice by supplementation of a developed optimized polyherbal formulation

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Abstract
This study evaluated in vivo anti-diabetic and anti-obesity activity of a polyherbal formulation's methanolic extract containing an optimized ratio of edible seeds (*Salvia hispanica*, *Chenopodium quinoa*, *Nelumbo nucifera*). Diet-induced obese mice model (C57BL/6) was developed by feeding the mice a high-fat diet for 10 weeks resulting in hyperglycemia and obesity. Different doses (125, 250 and 500 mg/kg of body weight) of formulation were administered orally daily for 6 weeks. Fasting blood glucose and body weight were monitored throughout the study. At the end of the study, serum parameters were analyzed and histological examinations were performed. There was a significant reduction in fasting blood glucose levels and body weight in animal groups receiving polyherbal formulation. Lipid profile was improved as revealed by a reduction in serum triglycerides and total cholesterol. Histological study showed an improvement in liver, kidney and pancreatic sections of treated mice. High-performance thin layer chromatography was performed to identify the phytochemicals responsible for the above-mentioned bioactivities. The results revealed the presence of flavonoid (rutin) in seeds of *N.nucifera* and in the polyherbal formulation. For the first time, this study demonstrated the anti-diabetic and anti-obesity potential of the optimized formulation. The formulation can be used as a potential therapy for management of diabesity.

Keywords Anti-diabetic · Anti-obesity · Polyherbal formulation · Diet-induced obese mice · Edible seeds · HPTLC

Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and hyperlipidemia as a result of altered carbohydrate and lipids metabolism occurring due to defects in insulin secretion, insulin action or both (Ojiaco et al. 2016; Ben Salem et al. 2017). In recent years, sedentary lifestyle and consumption of calorie-rich diet has resulted in increased incidence of diabetes and obesity also known as diabesity. Consumption of calorie-rich diet causes abnormal lipid metabolism, increased production of reactive oxygen species and oxidative stress, development of hyperinsulinemia and insulin resistance resulting in secondary diabetic complications (Bansal et al. 2012; Tan et al. 2018; Kopp 2019). Thus, considering these conditions, the treatment of type 2 diabetes mellitus (T2DM) should focus on modulating both glucose and lipid metabolism (Ben Salem et al. 2017).

There are various synthetic drugs available for treatment of T2DM, out of which intermediate-acting insulin, glibenclamide and metformin are accepted in almost all countries (Bazargani et al. 2014). These drugs are helpful in the management of T2DM, but there is no drug available for holistic management of T2DM (Rawat et al. 2013). Additionally, the adverse effect associated with currently available synthetic medicines has created the necessity for finding alternatives, mainly from natural sources, as these are easily available and possess less or even no side effects (Tran et al. 2020).

Herbal formulations developed from versatile combinations of pharmacologically potent herbs are being used in the treatment of several ailments including diabetes and obesity (De et al. 2019). The safety and efficacy of these herbs in producing multiple target action have made them a potential source of treatment for T2DM (Chang et al. 2013). Among various natural sources for T2DM management, some edible seeds (*Salvia hispanica, Chenopodium quinoa, Nelumbo*

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nucifera) have gained attention as super foods/functional foods (food exhibiting therapeutic effects apart from providing nutrition). *S. hispanica* (Chia) seeds are commonly consumed as salad sprouts, beverages, cereals, salad dressing or eaten raw (Kulczy´nski et al. 2019). Polyphenols from *S.hispanica* have been reported to be potent inhibitors of α-glucosidase and pancreatic lipase with antioxidant potential (Marineli et al. 2014; Martínez-Cruz and Paredes-López, 2014; Rahman et al. 2017; Rubavathi et al. 2020). Furthermore, *S.hispanica* seeds also improved glucose and insulin tolerance and reduced adiposity in animal models (Rosario et al. 2020; Enes et al. 2020; Oliva et al. 2021). Consumption of the seeds has shown to exhibit potent activities against dyslipidemia, cardiovascular disease, glucose homeostasis and insulin resistance in T2DM patients (Vuksan et al. 2017a, b; Cassia et al. 2022). *C. quinoa* (Quinoa) is known as "golden grain" and has been exploited in the development of nutrient-enriched novel food products, gluten-free products and can be eaten as rice replacement, soups, yogurt, salads, popcorn, flour and even sprouted (Brend et al. 2012; Angeli et al. 2020; Del Hierro et al. 2020). Recently, *C. quinoa* has gained attention due to its high nutritional value, phytochemical content and therapeutic activities including anti-hyperglycemic and lipase inhibitory potential (Graf et al. 2014; Tang et al. 2016; Lin et al. 2019; Song et al. 2021a, b; Oluwagunwa et al. 2021; Chen et al. 2022). Additionally, antioxidant potential of the seeds has also been reported (Park et al. 2017; Liu et al. 2020b, a; Sampaio et al. 2020; Enciso Roca et al. 2021; García-Parra et al., 2021). *N.nucifera* (lotus) seeds exhibit various flavonoids and alkaloids which contribute to its antioxidant and anti-diabetic potential (Rai et al. 2006; Pan et al. 2009; Kim and Shin 2012; You et al. 2014; Zhu et al. 2017). The seeds have been reported to exhibit hepatoprotective and anti-obesity activity (Sohn et al. 2003; Raajeswari and Meenakshi 2017; Wang et al. 2019).

Since diabesity is a multi-variant metabolic disorder, a single herb may be insufficient to exhibit the desired therapeutic effect. Just as combinational dosage forms of synthetic drugs are promoted in the treatment of T2DM with a multidimensional approach, polyherbal formulations (PF) have gained more attention over single herb for management of T2DM due to their synergistic therapeutic effects (Parasuraman et al. 2014; De et al. 2019). Various studies have reported the potential of PF in management of T2DM (Kiani et al. 2018; Virk et al. 2020; Pérez Gutiérrez et al. 2021; Perumal et al. 2022). Even though these studies proved the efficacy of herbal formulation treatment on glycemic control to be significant, their effect on lipid profile needs to be proved (Suvarna et al. 2021). Furthermore, identification of phytochemicals present in a newly developed herbal formulation is essential for supporting its therapeutic potential and to increase their acceptability prior to commercialization (Shinde et al. 2016). High-performance thin layer chromatography (HPTLC) is an established method for the analysis of various phytochemicals (Kustrin and Hettiarachchi 2014).

There are different reports to prove the anti-diabetic effect of these edible seeds, but there is no report demonstrating the anti-diabesity effect of methanolic extract of these three seeds combination on high-fat diet (HFD)-induced diabetic mice. For the first time, this research work reports the anti-diabesity effect of a PF, which was developed from methanolic extract of three edible seeds (*S.hispanica, C.quinoa, N. nucifera*) in a chemometrically optimized ratio with the aid of Design Expert Software 11.0 in a diet-induced obese (DIO) mice model (C57BL/6). The study also made an effort to identify the phytochemicals responsible for therapeutic effect of the PF using HPTLC.

Materials and method

Seeds collection

Seeds of *S. hispanica*, *C. quinoa* and *N. nucifera* were purchased from local shops (Bangalore) and authenticated at the Regional Ayurveda Research Institute for Metabolic Disorders (RARIMD), Bangalore, India. The authentication number for *S. hispanica*, *C. quinoa* and *N. nucifera* was RRCBI-mus189, RRCBI- mus213 and RRCBI-10394, respectively.

Preparation of polyherbal formulation

The individual seeds were powdered and mixed in a ratio of 1:1:1 according to earlier performed optimization studies (Tanisha and Majumdar 2019) and extracted with methanol using the Soxhlet apparatus. Extracts were then filtered through Whatman filter paper No. 1. The filtrates were concentrated by a rotary evaporator and the extracts were stored at 4 °C for further analysis. Methanol was chosen as a solvent for the preparation of PF extract, as methanol is reported to be the best solvent for achieving a maximum yield of phytochemicals due to higher solubility of these compounds (Truong et al. 2019; Kumar et al. 2020). Furthermore, studies have shown methanol as a choice of solvent for anti-diabetic studies (Chandran et al. 2017; Singh et al. 2018).

Animals

6-week-old male mice (C57BL/6) weighing 22 ± 3 g were obtained from In Vivo Biosciences, Bangalore, India. Mice were housed in polypropylene cages, maintained at 23 ± 1 °C, $60 \pm 10\%$ humidity, exposed to 12 h cycles of light and dark and had access to diet and water ad libitum

throughout the study. Before starting the experiment, the mice were allowed to acclimatize for 7 days.

Diet-induced obese (DIO) model development

After the acclimatization period was over, mice were fed either low-fat diet with 10% kcal fat (Research Diets Inc. D12450B) or a high-fat diet with 60% kcal fat (Research Diets Inc. D12492) for 10 weeks to develop the DIO mice model (Shang et al. 2017). Diet formulation for the study was based on nutrient to calorie ratio to ensure that all the diet contains the same level of energy. The composition of commercial diet used in the study is presented (Table 1). The hyperglycemic status of mice was confirmed by fasting glucose levels. Thereafter, the diet-induced obese mice were treated with different doses of PF for another 6 weeks (drug treatment period) with a continuation of high-fat diets. All animal experiment protocols were approved by In Vivo Biosciences Institutional Animal Ethical Committee in agreement with CPCSEA (Committee for Purpose of Control and supervision of Experiments on Animals) guidelines (Regd. No. 1165/PO/RcBiBt-S/NRc-L/08/CPCSEA, Proposal number 62/2018).

Table 1 Composition of low-fat and high-fat diet used in the study

Energy (kcal%)	Low-fat diet 10% kcal fat (D12450B)	High-fat diet 60% kcal fat (D12492))
Carbohydrate	70	20
Fat	10	60
Protein	20	20
Energy content (kcal)		
Corn starch	2160	0
Casein	800	800
$_{L}$-Cystine	12	12
Sucrose	1400	275
Soybean oil	225	225
Maltodextrin 10	140	500
Lard	180	2205
[a]Vitamin mix V10001	40	40
[b]Total	4507	4507

Two commercial diets were used in the study. The low-fat control diet contained 10% kcal fat (Research Diets Inc, D12450B) and the high-fat diet (HFD) contained 60% kcal fat (Research Diets Inc, D12492)

[*]Adapted from Research Diets Inc

[a]Vitamin mix contained: 4000 IU vitamin A acetate (500,000 IU/gm), 1000 IU vitamin D3 (100,000 IU/gm), 50 IU vitamin E acetate (500 IU/gm), 2 mg folic acid, 30 mg nicotinic acid, 0.5 mg menadione sodium bisulfite (62.5% menadione), 0.2 mg 1.0% biotin, 10 µg 0.1% cyanocobalamin, 16 mg calcium pantothenate, 7 mg pyridoxine–HCl, 6 mg riboflavin, 9.8 mg sucrose and 6 mg thiamine HCl for a total of 10 gm to be used at 10gm/kg or 10 gm/4000 kcal digestible energy

[b]The calculated dietary energy is 3.85 kcal per gram for the control diet and 5.24 kcal per gram for the HFD

Acute toxicity test

Acute oral toxicity test was performed by administering the methanolic extract of PF to C57BL/6 male mice having a weight of 25–30 g ($n=6$/group). The test was performed as per the Organization for Economic Co-operation and Development (OECD) guideline 423. Different doses of PF extract (125, 250, 500, 1000 and 2000 mg/kg) were suspended in 0.5% carboxyl methylcellulose (CMC) and administered orally to the HFD-fed mice groups as mentioned in earlier studies (Zhang et al. 2016; Sahu et al. 2018). The normal control group was treated with CMC suspension. Mice were observed initially for 4 h and then for14 days. The limit dose for the study was 2000 mg/kg b.wt., which did not show any toxic effects in the treated mice. Thus, 1/4th (500 mg/kg b.wt.), 1/8th (250 mg/kg b.wt.) and 1/16th (125 mg/kg b.wt.) of the limit dose were chosen for further anti-diabetic studies (Zhang et al. 2016; Sahu et al. 2018).

Grouping and dosing of animals

Male mice were chosen for the study including oral starch tolerance test, oral sucrose tolerance test, oral lipid tolerance test and anti-diabetic activity. It has been shown that HFD feeding for 14 weeks resulted in prominent hyperinsulinemia and alterations in islet size without significant changes in β-cell function in C57BL/6 male mice in comparison to female mice (Pettersson et al. 2012). Therefore, C57BL/6 male mice seem to be an appropriate model for studying metabolic syndromes such as diabetes and obesity.

In the oral starch tolerance test, oral sucrose tolerance test and oral lipid tolerance test mice were randomly divided into five groups ($n=6$ mice per group). For starch and sucrose tolerance test, Group I was treated with 1% CMC (1 ml/100 g of body weight (b.wt.)). Group II was treated with the standard drug, glibenclamide (5 mg/kg). Groups III, IV and V were treated with PF extract at a dose of 125 mg/kg, 250 mg/kg and 500 mg/kg, respectively.

For lipid tolerance test, Group I was treated with corn oil emulsion (5 ml/kg b.wt.). The composition of oil emulsion was a combination of cholesteryl oleate (2 mg), cholic acid (80 mg), saline (6 ml) and corn oil (6 ml). Group II was administered with orlistat (50 mg/kg). Groups III, IV and V were treated with PF extract at a dose of 125 mg/kg, 250 mg/kg and 500 mg/kg, respectively.

For the anti-diabetic study, mice were randomly divided into six groups (5 groups of diabetic mice and 1 additional group of normal mice, 6 mice per group) as shown below.

Group I: Normal mice treated with 1% CMC (1 ml/ 100 g b.wt.).

Group II: DIO mice: HFD without any treatment.

Group III: DIO mice: HFD + glibenclamide (25 mg/kg/b.wt.).

Group IV: DIO mice: HFD + PF extract 125 mg/kg b.wt.

Group V: DIO mice: HFD + PF extract 250 mg/kg/day b.wt.

Group VI: DIO mice: HFD + PF extract 500 mg/kg/day b.wt.

The DIO mice were treated with the above-mentioned dose of PF extract and standard drug (glibenclamide) for 6 weeks. Glibenclamide was selected as a standard drug based on reports of different studies (Tamiru et al. 2012; Belayneh and Birru 2018). The three doses of the PF extract were determined based on the results of the acute oral toxicity study and according to earlier reported studies (Shin and Yoon 2012; Sharma et al. 2014; Araújo et al., 2016; Sarega et al. 2016). The oral route of administration was used in the study because plants are taken orally (Belayneh and Birru 2018). All the doses were given using an oral gavage after dissolving the PF extract in CMC at a volume of 1 ml/100 g body weight of the mice.

Oral carbohydrate challenge tests

The oral carbohydrate tolerance tests were conducted according to standard protocol, which has been discussed below. The tests were carried out using starch and sucrose in normal and diabetic mice, respectively.

Oral starch tolerance tests

The oral starch tolerance test was conducted according to standard protocol (Ye et al. 2002). Mice were kept on fasting for 16 h before the experiment, but had access to water ad libitum. Mice were divided into five groups ($n = 6$) and treated as described above. 30 min after dosing, the mice were loaded with corn starch (3 g/kg b.wt.) with an oral gavage. Blood was taken from tip of the tail vein at 0, 0.5, 1 and 2 h. Blood glucose concentration was determined by Accu-Chek glucometer (Roche Diagnostics) according to earlier reported studies (Mudgal et al. 2016; Tang et al. 2018).

Oral sucrose tolerance test

The oral sucrose tolerance test was carried out with a similar protocol mentioned in the oral starch tolerance test (Ye et al. 2002). However, instead of starch, the mice were loaded with sucrose at a dose of 4 g/kg. Blood glucose concentration was determined by Accu-Chek glucometer (Roche Diagnostics).

Oral lipid tolerance test

Oral lipid tolerance test (OLTT) was conducted as per the standard protocol (Zhang et al. 2008; Ghelani et al. 2017). The animals were kept fasting for 16 h before the experiment, but had access to water ad libitum. Mice were divided into five groups ($n = 6$) and treated as described above. 30 min after dosing, the mice were loaded with corn oil emulsion (5 ml/kg b.wt.) with an oral gavage. Blood was taken from the tip of the tail vein at 0, 1.5, 3 and 4 h. The level of plasma triglyceride (TG) was measured using the Accutrend® Plus system (Roche).

Assessment of anti-diabetic activity of the extract in DIO mice

For anti-diabetic evaluation of the PF extract, mice were fasted overnight (16 h). Mice were divided into six different groups ($n = 6$). Then the animals were treated according to their respective groups as mentioned above. The animals were supplemented with PF (via oral gavage) every day at the same time (9AM–10 AM) till the end of the study (6 weeks). For blood glucose measurement, the blood was obtained from the tip of the tail and measured using the Accu-Chek glucometer (Roche Diagnostics) till the end of the study. The blood glucose level of the treated animals was measured once a week throughout the study, i.e., for 6 weeks. The body weight of the animals was measured on a weekly basis throughout the study. On completion of the study, mice were fasted overnight and blood was obtained from the tip of the tail by pricking the tail and the TG level was measured using Accutrend Plus (Roche) triglyceride meter. Blood was drawn by cardiac puncture. Blood was collected in the microfuge tube, allowed to stand for 30 min at room temperature and centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a fresh tube and stored at −80 °C until further use.

Histological studies

Vital organs (kidney, liver and pancreas) were excised and stored in cold 10% formalin containing phosphate-buffered saline for at least 24 h. Tissues were embedded in paraffin wax and 4 µm sections of tissues were stained with hematoxylin and eosin (H&E). Tissue sections were observed using an inverted microscope (Nikon Eclipse TE2000-5, Japan) at a magnification of 100× to find the morphological changes in treated and untreated groups.

HPTLC

HPTLC was performed using standard protocol (Varkey and Kasthuri, 2016). The standard rutin was dissolved in

methanol (1 mg/ml). 10 mg of *S.hispanica*, *C.quinoa* and *N.nucifera* seed extracts and PF extract were dissolved in 1 ml of methanol. HPTLC was performed on silica gel $60F_{254}$, 200X100 mm HPTLC plates (Merck, Darmstadt, Germany) with ethyl acetate:formic acid:acetic acid:water (14.4:1.4:1.4:2.8 (v/v) as a mobile phase. Standard solution rutin (10 µl) and test solution (10 µl) were loaded as 5 mm band length on HPTLC plate with the help of a CAMAG LIWOMAT 5 sample applicator at a distance of 10 mm from the edge of the plates. The sample-loaded plate was kept in a twin trough glass chamber (saturated with mobile phase for 30 min). The plate was developed in mobile phase up to 90 mm. The developed plate was dried in hot air to evaporate the remaining solvents. The plate was kept in a photo-documentation chamber (CAMAG REPROSTAR 3) to capture the images in white light, UV 254 nm and UV 366 nm. For derivatization, the plates were sprayed with a specific natural product reagent (1 g of 2-aminoethyl diphenylborinate in 200 ml of ethyl acetate). The plate was heated at 110º C for 3 min, then after cooling the plates were kept in photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured at 254 nm and 366 nm and white light. The peak table, peak densitogram and peak display were recorded. The retention factors (R_f) and % area were calculated by the WIN CATS software (version 4X).

Statistical analysis

Experimental values were expressed as mean ± SD ($n = 6$). Statistical analysis was performed using the one-way analysis of variance (ANOVA), followed by Dunnett's test in GraphPad Prism 6.0. Results were considered statistically significant at $p < 0.001$ (Gao et al. 2016).

Results and discussion

Toxicological studies

During the observation period, no abnormality and mortality in the behavior of animals was seen at a dose of 2000 mg/kg.

DIO model development

HFD supplementation resulted in increased body weight (~two fold) of DIO mice compared to lean control. Serum TG was significantly high compared to the lean control. 10 weeks of HFD supplementation resulted in impaired glucose tolerance in mice. It has been reported that the DIO model mimics the progression of impaired carbohydrate metabolism, increased hepatic glucose production, insulin resistance, compromised β-cell function, obesity and diabetes when fed with HFD as seen in humans (Kakimoto and Kowaltowski 2016).

Oral starch and sucrose tolerance test

Herbal extracts have been shown to control the elevation of starch and sucrose-associated postprandial blood glucose levels by inhibiting α-amylase and α-glucosidase enzymes. Thus, these extracts influence the absorption and metabolism of starch and sucrose (Subramanian et al. 2008). Similar to the above-mentioned literature, the present study showed that PF administration lowered elevated blood glucose levels in starch- and sucrose-fed DIO mice. PF extract significantly ($p < 0.001$) lowered elevated blood glucose levels in a dose-dependent manner when compared to diabetic control which received only starch and sucrose solution, respectively, for starch and sucrose tolerance test (Fig. 1 and Fig. 2). Also, at 2 h the blood glucose-lowering effect of the middle and higher dose (250 and 500 mg/kg b.wt.) of PF was comparable to glibenclamide. It can be said that PF delays the digestion of starch and sucrose, thus, lengthening the time needed for carbohydrate absorption. The tendency of the PF to suppress the increased blood glucose levels in starch-loaded HFD mice suggests the involvement of α-glucosidase inhibition as mentioned in earlier studies (Subramanian et al. 2008). Different studies have performed oral starch and sucrose tolerance tests for single herbs (De La Garza et al. 2014; Dakam et al. 2021; Solares-Pascasio et al. 2021),

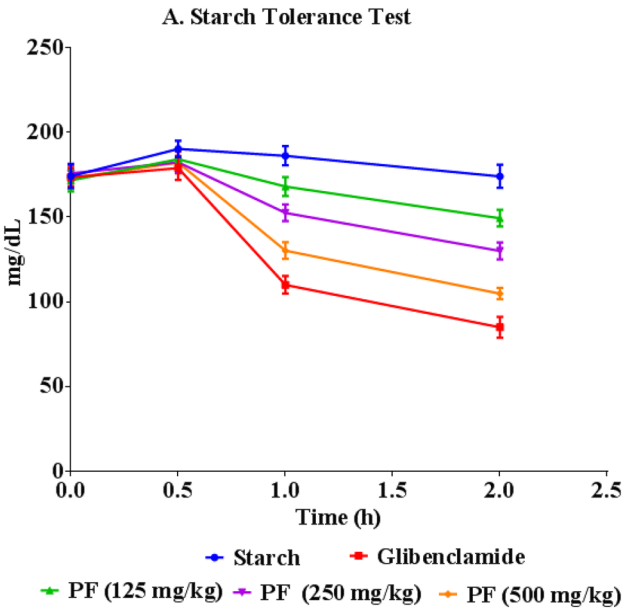

Fig. 1 Effects of PF on oral starch tolerance over a 2 h period in diabetic mice. Values were expressed as mean ± SEM ($n = 6$), compared with the control. Values were statistically significant at $p < 0.001$. *PF* polyherbal formulation

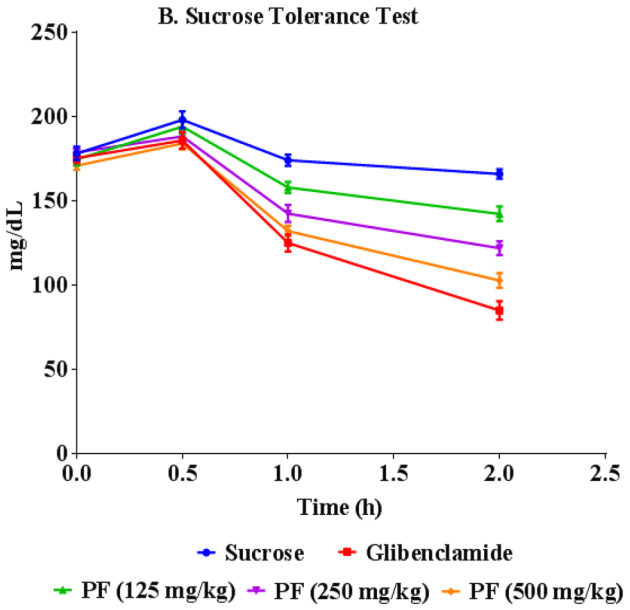

Fig. 2 Effect of PF on sucrose tolerance over a 2 h period in diabetic mice. Values were expressed as mean ± SEM ($n = 6$), compared with the control. Values were statistically significant at $p < 0.001$. *PF* polyherbal formulation

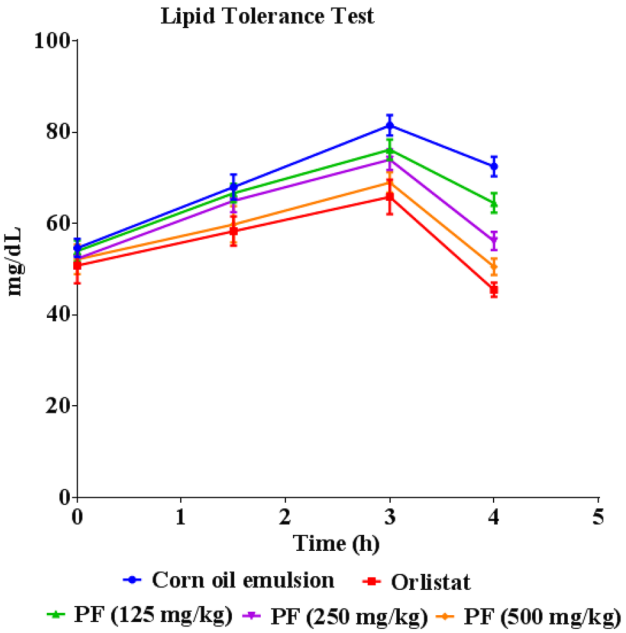

Fig. 3 Effect of PF on oral lipid tolerance of diabetic mice over a 4 h period. Values are expressed as mean ± SEM ($n = 6$), compared with the control. Values are statistically significant at $p < 0.001$. *PF* polyherbal formulation

but rarely any reports available for oral starch and sucrose tolerance tests of a PF. To our knowledge, the present study for the first time demonstrates α-amylase and α-glucosidase inhibitory potential of the optimized PF methanolic extract using oral starch and sucrose tolerance test.

Oral lipid tolerance test

OLTT was performed as this assay evaluates lipid metabolism as well as whole-body lipid metabolism. The data from OLTT can be used to access information about the lipase inhibitory activity of pancreas and intestinal lipid absorption when investigating the suppressive effects of a herbal formulation's extract on postprandial hypertriglyceridemia. Thus, it can be said that OLTT is a useful method to evaluate lipid metabolism (Ochiai 2020). Increased level of lipids is known as one of the major risk factors for obesity, hypercholesterolemia, atherosclerosis and myocardial infarction (Kim et al. 2018). In the present study, elevated plasma TG levels were observed in mice receiving corn oil emulsion alone at 4 h. Standard drug orlistat (5 mg/ kg) was used for this assay. The drug is commonly used to reduce the weight of overweight adults (Drew et al. 2007). Treatment with orlistat showed a reduction in TG when compared with HFD-fed group of animals. The PF extract (250 and 500 mg/kg b.wt.) significantly ($p < 0.001$) suppressed incremental plasma TG at 3 and 4 h (Fig. 3). The TG suppressing potential of the PF was comparable to standard drug (orlistat) used in the study. The results of the study indicated that administration of PF extract suppressed the intestinal TG absorption in OLTT, which can ameliorate hyperlipidemia as well as obesity-related parameters in HFD-fed obese mice. This finding of the study is important, as there is rarely any PF studied for OLTT. The study may also be helpful in selecting appropriate animal model and standard protocols for OLTT.

Glycemic status

The increase in fasting blood glucose level is an important characteristic of T2DM (Kifle and Belayneh 2020). Anti-hyperglycemic activity of PF in comparison to glibenclamide was estimated by measuring blood glucose levels on weekly basis. C57BL/6 on HFD feeding tended to have a slower glucose clearance compared to other groups. The PF extract at a dose of 250 and 500 mg/kg was found to be effective in lowering blood glucose levels. Furthermore, at a lower dose (125 mg/kg), PF extract did not show any significant effect on the blood glucose level. The glycemic control was nearly similar in animal groups treated with glibenclamide (5 mg/kg) and PF extract (250 and 500 mg/kg b.wt.). The decrease in fasting blood glucose level of PF treated DIO mice was observed from the 4th week and continued till the end of the study, i.e., for 6 weeks. The data obtained were comparable to that of the standard drug, glibenclamide (Fig. 4). The improvement in blood glucose levels observed in the present study correlated with the results obtained from the oral starch and sucrose tolerance

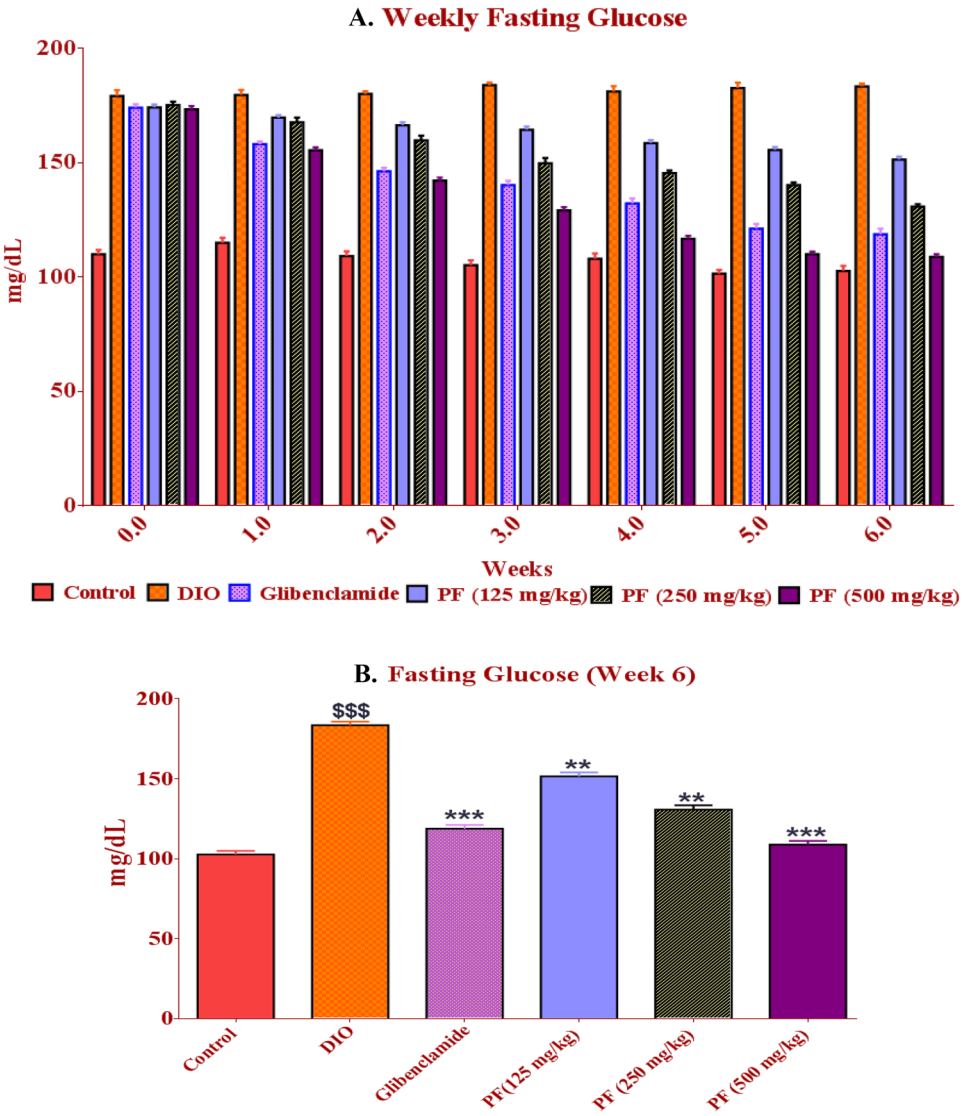

Fig. 4 Effect of PF supplementation on blood glucose level (mg/dL) in mice for weeks 1–6 (**A**) and specifically on the 6th week (**B**). Values represent mean ± SEM ($n = 6$). $^{**}p < 0.001$ and $^{***}p < 0.01$ as compared with DIO control. $^{\$\$\$}p < 0.01$ as compared with the control. Data were analyzed with one-way ANOVA followed by post hoc analysis (Dunnett's multiple comparison test). *PF* polyherbal formulation

and the same was reported in an earlier study (De La Garza et al. 2014). The anti-diabetic effect of herbal formulations (containing 3 herbs) has been reported earlier and the results of this study were similar to those reported in an earlier study, which has been discussed. A herbal formulation Gyeongshingangjeehwan 18 (composed of 3 herbs *Ephedra sinica*, *Laminaria japonica* and *Rheum palmatum)* showed anti-diabetic effect in HFD-fed mice when treated for 13 weeks. The formulation was given at a dose of 125, 250 and 500 mg/kg b.wt., out of which the higher dose (500 mg/kg b.w) inhibited hyperglycemia and hyperinsulinemia and improved glucose and insulin tolerance (Jang et al. 2018). A herbal formulation (C-DM4) containing a combination of *Coptidis rhizome*, *Salviae miltiorrhizae radix* and *cinnamomi cortex* was evaluated for its anti-diabetic and anti-obesity potential in HFD-fed mice. C-DM4 supplementation significantly reduced the increased levels of glucose, insulin, total cholesterol (TC), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in HFD-fed mice. In addition, C-DM4 extract inhibited lipid droplet accumulation in liver tissues of obese mice, hyperplasia of pancreatic islets and enlargement of adipocytes in adipose tissues (Jung et al. 2021). These results of the present study support the earlier findings that a combination of herbs or seeds offers better anti-hyperglycemic properties in comparison to individual herb or seeds.

Effect of PF on body weight

Consumption of more dietary fat may be related to increased fat accumulation and thus increased body weight (Yang et al. 2014; Lee et al. 2019; El-Zayat et al. 2019). Mice fed with HFD showed a significant increase in body weight compared to normal diet, as HFD consumption is considered the obesity hallmark (Meriga et al. 2017). Obesity is the main modifiable risk for T2DM. In the present study, the

body weight of PF-administered animal group showed a significant ($p < 0.001$) reduction in the body weight at a dose of 250 and 500 mg/kg respectively, when compared with the HFD-fed animal group (Fig. 5). Furthermore, at lower dose (125 mg/kg) PF extract showed non-significant effect on the body weight. The finding of this study is in line with previous reports which stated that herbal formulation control weight gain in HFD-fed mice. Tongbi San, a formulation comprising a combination of three herbs (*Cyperus rotundus* L., *Citrus unshiu Markovich* and *Poria cocos*) was found to exhibit anti-obesity potential in HFD-fed mice when supplemented for 11 weeks (Park et al. 2019). Another anti-obesity formulation named LI85008F (*Moringa oleifera, Murraya koenigii and curcuma longa*) prevented body fat accumulation and altered lipid metabolism in HFD-fed mice when supplemented for 13 weeks (Choi et al. 2021). Previous studies have reported that herbal formulation extract supplementation may prevent or improve obesity and insulin resistance by modulating lipid metabolism and suppressing appetite (Lee et al. 2016; Yimam et al. 2016).

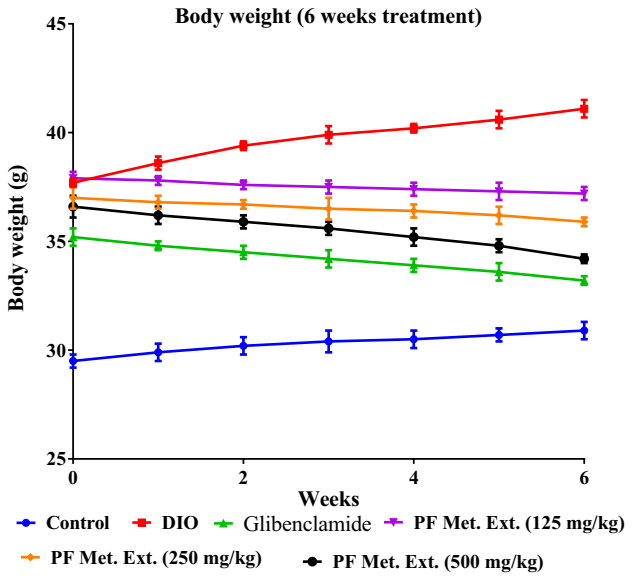

Fig. 5 Effect of PF supplementation on body weight of mice for weeks 1–6. Values are expressed as mean ± SEM ($n = 6$). Values are statistically significant at $p < 0.05$. *PF* polyherbal formulation

Effect on lipid profile

Lipid profile is crucial in the diagnosis and treatment of several diseases including T2DM. Diabetes mellitus-induced hyperlipidemia occurs as a result of the excess mobilization of fats from the adipose tissue due to the underutilization of glucose (Akpan et al. 2012). The elevated total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-c) and low levels of high-density lipoprotein cholesterol (HDL-c) in diabetic conditions imply diabetic dyslipidemia, which is caused due to insulin resistance leading to impairment of the key enzymes and pathways involved in lipid metabolism (Table 2) (Gao et al. 2009; Ozder 2014). There is a strong link between lipid metabolism and glucose metabolism and the inability to maintain lipid levels is a major cause of chronic complications in T2DM (Song et al., 2021a, b). In addition to this, the majority of T2DM patients exhibit dyslipidemia which is important in mediating the cardiovascular risk in T2DM (Parhofer 2015). Therefore, regulation of lipid metabolism should be considered as an important criterion along with glucose-regulating function in T2DM patients (Song et al., 2021a b). In the present study, the serum lipid profile was examined at the end of the study. A significant increment in lipid profiles of HFD-fed diabetic mice was observed including TC, TG and LDL-c, all this combined with a decrease in HDL-c. Middle and higher doses of PF extract (250 and 500 mg/kg) markedly improved lipid profile alterations induced by HFD, compared to low doses (125 mg/kg). Thus, it can be said that the anti-hyperlipidemic activity of PF extract was dose dependent. However, HDL-c level was not significantly different among the treated groups. This finding of the present study is in line with earlier reports which demonstrated that the TG, TC and LDL-c level reducing potential of herbal

Table 2 Effect of PF supplementation on serum lipid profile

Groups	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	TC (mg/dL)
Normal control	53.40 ± 0.41	36.46 ± 0.95	95.0 ± 0.73	143.1 ± 0.9
Diabetic control	25.80 ± 0.46$^{\$}$	91.78 ± 0.80$^{\$}$	135.71 ± 1.1$^{\$}$	259.6 ± 3.7$^{\$}$
Glibenclamide	38.70 ± 0.66*	80.83 ± 0.94*	134.04 ± 0.93	262.3 ± 2.0
125 mg/kg b.w	24.51 ± 0.66	81.16 ± 0.90*	115.50 ± 1.18*	233.0 ± 1.7*
250 mg/kg b.w	24.15 ± 0.86	80.39 ± 0.84*	112.12 ± 1.24*	228.3 ± 1.1*
500 mg/kg b.w	35.92 ± 0.87*	70.13 ± 0.41*	111.50 ± 1.61*	209.2 ± 2.3**

Values represent mean ± SEM ($n = 6$). *$p < 0.05$,**$p < 0.001$ as compared with DIO control. $^{\$}p < 0.05$, as compared with the control. Data were analyzed with one-way ANOVA, followed by post hoc analysis (Dunnett's multiple comparison test)

PF polyherbal formulation, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *TG* triglyceride, *TC* total cholesterol

extracts might be due to the presence of polyphenolic compound, which plays a role in the prevention of advanced glycation end product (AGE) formation in diabetic mice (Jia et al. 2009). It has been reported that the extract of herbal formulations may delay the absorption of glucose and fatty acids, thus providing few substrates for triglyceride production. In addition, the PF extract might have inhibitory effects on pancreatic lipase as demonstrated by OLTT, which may contribute to its anti-hyperlipidemic activities (Subhasree et al., 2015; Kifle and Belayneh 2020). The results of the present study were found to be similar to the earlier studied anti-obesity PF, Yangkyuksanwha-tang. The supplementation of Yangkyuksanwha-tang to HFD-fed mice for 6 weeks resulted in lower TC, TG and LDL-c levels with a reduction in body weight (Koh et al. 2019). Furthermore, a study demonstrated that herbal extract and glibenclamide might act on reserved fats and inhibit the release of free fatty acids, decreasing the TC and TG levels and increasing HDL levels and similar findings were observed in the present study (Rahman et al. 2021).

Liver histology

Liver is the vital organ that regulates the metabolism of glucose, lipid and proteins (Park et al. 2015). Obesity due to HFD leads to the accumulation of lipid in liver resulting in fatty liver and other health complications including T2DM. In the present study, histological changes in the liver were studied to understand the effect of PF on the liver of untreated and treated mice (Fig. 6). Administration of PF extract resulted in a reduction of lipid droplets accumulation and restoration of hepatic architecture in mice as evidenced by histological examination of the liver tissue. Liver of untreated mice appeared normal without inflammation and lipid accumulation. The results of histological study showed that PF administration reduced excessive lipid deposition in liver tissues by accelerating lipid metabolism in T2DM mice and maintained the normal lipid metabolism, which played an effective protective role for the liver. This finding of the study is important because it has been reported that lipids and glucose play an important role in energy metabolism and both are regulated by the liver, thus exhibiting a close relationship between glucose and lipid metabolism (Parhofer 2015).

Different studies have shown that herbal formulation consisting of three or more herbs improve fatty liver condition in DIO mice. A herbal formula named Yin Zhi Huang consisting of four herbs (*Artemisia scoparia*, *Gardeniae fructus*, *Scutellaria baicalensis Georgi* and Lonicerae *japonicae flos*), used as food in Asia, was found to be effective in controlling hepatic steatosis. The formulation was fed to C57BL/6 male mice for 16 weeks along with HFD, which resulted in a reduction of body weight, alleviation of hepatic lipid accumulation and restoration of plasma levels of TG and TC. The study suggested that supplementation of the formulation ameliorates diet-induced obesity and hepatic steatosis by decreasing AMPK/SREBP-1 pathway-mediated de novo lipogenesis and increasing AMPK/ACC/CPT1A pathway-mediated mitochondrial fatty acid β oxidation (Yao et al. 2020). Another herbal composition GGEx18, composed of *Laminaria japonica*, *Rheum palmatum* and *Ephedra sinica*, was able to prevent hepatic steatosis and hyperlipidemia at a dose of 250 and 500 mg/kg b.wt. in HFD-fed C57BL/6 mice by activating hepatic PPARα (Shin and Yoon 2012). Similar to our study, the lower dose of

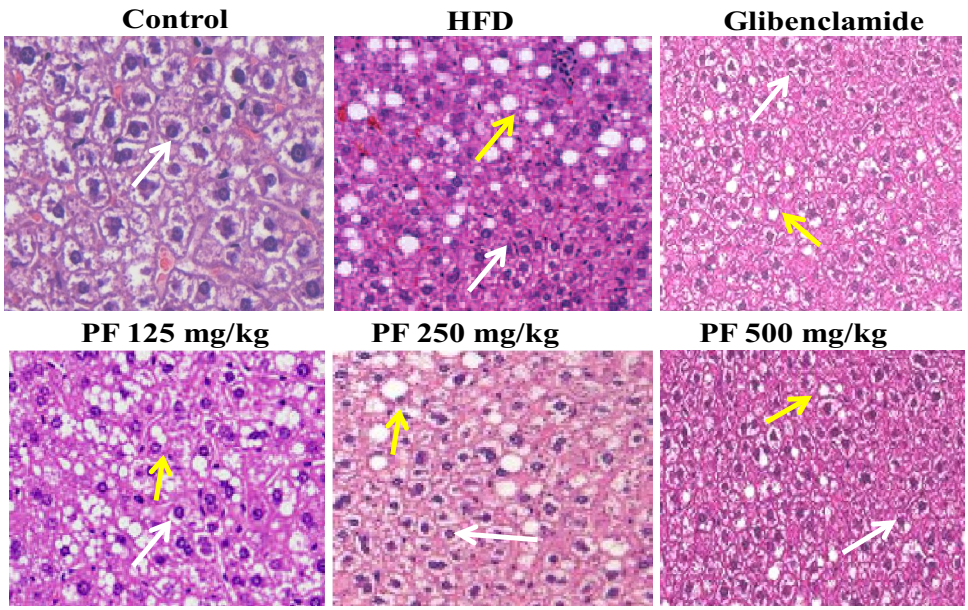

Fig. 6 Histological examination of liver tissue (magnification, × 100) after hematoxylin and eosin staining. In the control group, HFD group and other treated groups lipid droplets are indicated by yellow-colored arrows, whereas hepatocytes in all the groups are indicated by white-colored arrows. *HFD* high-fat diet-induced obese diabetic mice, *PF* polyherbal formulation

GGEx18 (125 mg/kg b.wt.) did not show any significant changes in liver of HFD-fed mice, proving that doses of 250 and 500 mg/kg b.wt. were effective in controlling hepatic steatosis. In another study, C57BL/6 male mice were fed HFD for 16 weeks and then treated with a herbal formula, MIT (*Ephedra sinica*, *Panax ginseng* and *Alisma orientale*), for 8 weeks. The results of the study suggested that MIT has the potential to prevent and treat obesity-related non-alcoholic fatty liver disease via regulating the levels of serum glucose and free fatty acids, inflammation, lipid accumulation and ROS-mediated liver damage (Ahn et al. 2020). The results of our study are in agreement with the above-mentioned studies that herbal formulation improves fatty liver by effectively reversing metabolic and histological changes associated with HFD consumption.

Kidney histology

HFD supplementation has been reported to cause alteration in kidney lipid metabolism, leading to renal damage including glomerulosclerosis, interstitial fibrosis, albuminuria and increased oxidative stress ultimately causing T2DM and its complications (Deji et al. 2009; Garcia et al. 2018). In the present study, the control group showed normal kidney architecture (Fig. 7). In HFD-fed obese and diabetic mice, kidneys showed increased Bowman's space, mild swelling of the glomerulus and tubular degeneration. Administration of PF extracts to HFD + diabetic mice for 6 weeks resulted in an alleviation of glomerulus swelling and normal glomerulus architecture. Similar findings were reported for a potent anti-obesity formulation 18KHT01 when studied on HFD-fed C57BL/6 male mice. The formulation consisted of *Quercus acutissima*, *Camellia sinensis* and *Geranium thunbergii*, along with *Citrus limon* (fruit juice). Supplementation of 18KHT01 ameliorated histological alterations of kidney, which included mild infiltration of macrophages and enlargement of Bowman's space of glomeruli (Pandeya et al. 2021). The findings of the present study may provide information on renal changes in HFD-fed mice, as very few herbal formulations have been studied for their effect on renal changes in HFD-fed mice.

Pancreas histology

In an excessive nutritional state, as seen in obesity, hyperglycemia and hyperlipidemia are often noticed, favoring insulin resistance and chronic inflammation. The differences in genetic susceptibility of β-cells result in toxic pressures including inflammation, inflammatory stress, ER stress, and metabolic, oxidative and amyloid stress, with the potential of ultimately leading to the loss of islet integrity (Fraulob et al. 2010; Galicia-Garcia et al. 2020). Our results corroborate these findings and showed that HFD supplementation caused loss of islet integrity, which was indicated by the irregular shape of pancreatic islet. After administration of PF for 6 weeks, improvement in the pancreatic tissues was observed, which is evidenced by more integrated cell structure in the pancreatic islets, with normal shape and size (Fig. 8). Apart from this, no major changes were observed in HFD-fed mice and this finding is in agreement with earlier finding (Lu et al. 2020). The PF showed dose-dependent improvement in tissue architecture. The standard drug (glibenclamide) used for the study has been reported to improve the architectural form of the islets of Langerhans and enhance the insulin

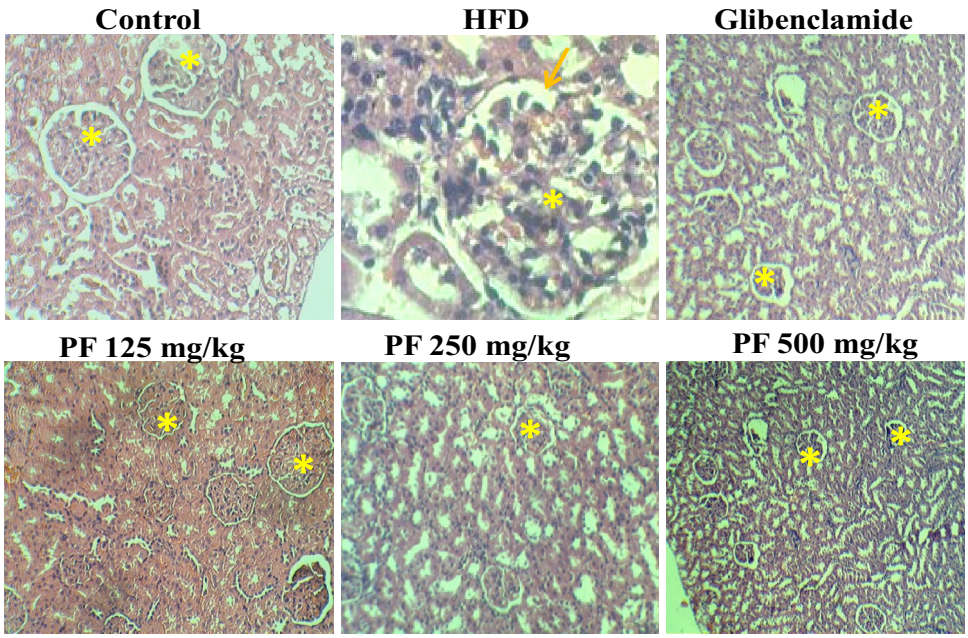

Fig. 7 Histological examination of kidney tissues (magnification × 100), after hematoxylin and eosin staining. Asterisk mark indicate glomerulus, whereas arrow in HFD group denotes increased Bowman's space. *HFD* high-fat diet-induced obese diabetic mice, *PF* polyherbal formulation

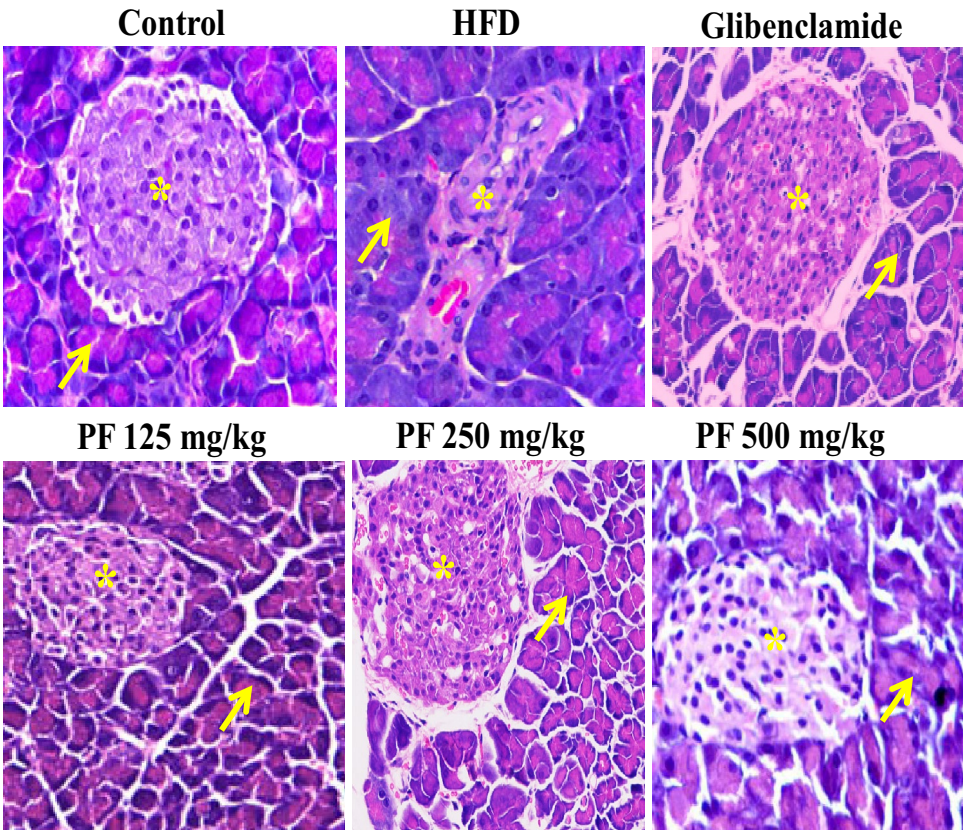

Fig. 8 Histological examination of pancreas (magnification × 100) after hematoxylin and eosin staining. The arrows indicate acini (arrow) and the asterisk mark indicate islet of langerhans in pancreas. *HFD* high-fat diet-induced obese diabetic mice, *PF* polyherbal formulation

secretion from the pancreatic beta cells (Asgary et al.2012; Parasuraman et al. 2019). The histological findings of this study were in accordance with earlier studies which suggested that the pancreas protective potential of PF could be due to reduction of oxidative stress (mediated by phenolic compounds present in PF), thus preserving pancreatic β-cell integrity and leading to insulinotrophic action (Taghizadeh et al. 2015). Earlier studies have shown that a combination of grape pomade and omija fruit preserved pancreatic islets' architecture with increased pancreatic expressions of insulin and glucagon in HFD-fed mice (Cho et al. 2015). Relating earlier reports to our current findings, it can be said that the pancreas protective effect of PF can help in combating T2DM and its secondary complications. The anti-diabetic and anti-hyperlipidemic potential of PF observed in this study could be due to the increased amount of phytochemical constituents resulting from combining the three seeds in an optimized ratio.

HPTLC

HPTLC analysis of three individual seeds and PF was conducted to identify the phytochemicals of therapeutic importance. The presence of flavonoid (rutin) was confirmed in *N.nucifera* and PF with R_f values of 0.37 after derivatization with natural product reagent under UV light at 254 and 366 nm and in white light (Figs. 9, 10, 11). Rutin is known to exhibit various health-beneficial properties such as antioxidant, anti-inflammatory, anti-carcinogenic and anti-hyperglycemic effects (Kamalakkannan and Prince, 2006; Jadhav and Puchchakayala, 2012; Hunyadi et al. 2012; Panche et al. 2016; Prasad and Prasad 2019). Different unknown compounds were found in the seed extract of *S.hispanica*

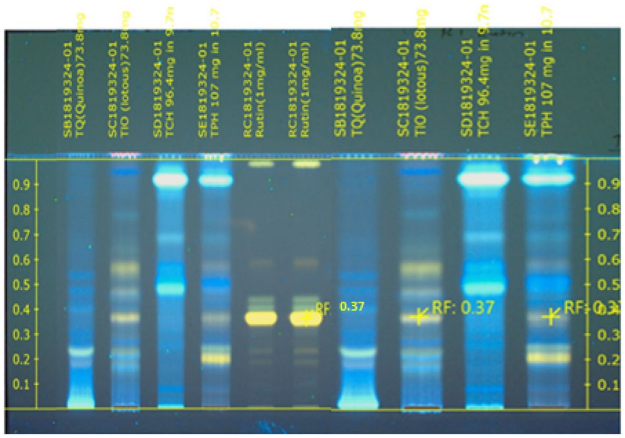

Fig. 9 HPTLC fingerprinting profile of PF and individual seeds with standard (rutin) at 366 nm * *TQ Chenopodium quinoa*; *TLo Nelumbo nucifera*; *TCH Salvia hispanica*; *TPH* polyherbal formulation

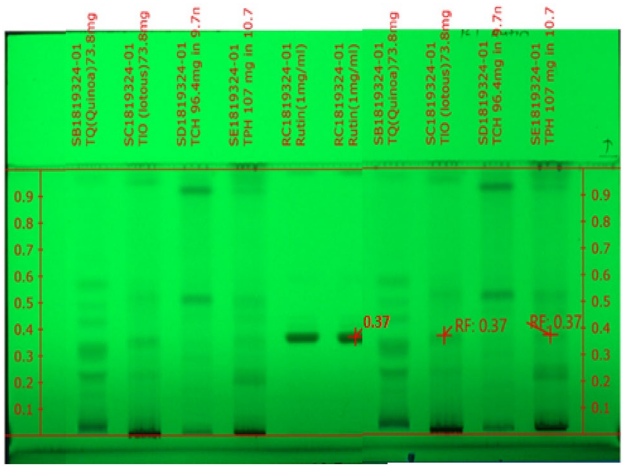

Fig. 10 HPTLC fingerprinting profile of PF and individual seeds with standard (rutin) at 254 nm * *TQ Chenopodium quinoa*; *Tlo Nelumbo nucifera*; *TCH Salvia hispanica*; *TPH* polyherbal formulation

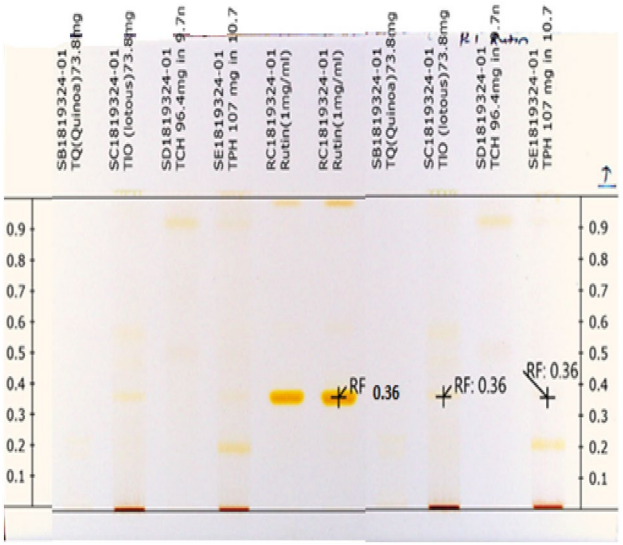

Fig. 11 HPTLC fingerprinting profile of PF and individual seeds with standard (rutin) in white light * *TQ Chenopodium quinoa*; *Tlo Nelumbo nucifera*; *TCH Salvia hispanica*; *TPH* polyherbal formulation

and *C.quinoa*. Studies have found that flavonoids not only possess lipase inhibitory potential, but also α-glucosidase inhibitory activity, which can prevent hyperglycemia, alleviate hyperinsulinemia, increase glucose tolerance and prevent and treat obesity (Liu et al. 2020b, a). Flavonoids from *N.nucifera* leaf showed anti-hyperglycemic effect in diabetic mice (Zhou et al. 2009). Flavonoids are also considered as α-amylase inhibitors (Najafian et al, 2010). Furthermore, studies have shown that HPTLC analysis is useful in identifying phytochemicals including flavonoids in various seeds and PF which exhibit anti-hyperglycemic effects (Cao et al. 2003; Parimala and Shoba, 2014; Salunke et al. 2015; Bhardwaj and Modi, 2017; Kumar et al. 2021). The outcomes of this study were in line with the formerly published studies which reported that the possible anti-hyperglycemic and anti-hyperlipidemic action of flavonoid in single and combinatorial herbal formulation extract in HFD-fed rats was found to be upregulation of hepatic superoxide dismutase activity, reduction of hepatic malondialdehyde content, downregulation of hepatic CYP2E1 expression and increase of glucose transporter 4 expressions in skeletal muscle of the treatment-receiving rats (Ma et al. 2012; Ojiaco et al. 2016). The developed PF could effectively reduce postprandial free fatty acid which could be attributed to its phenolic constituents as these are reported to inhibit pancreatic lipase by competitively binding to the enzyme active site (Yoshikawa et al. 2002; Martinez-Gonzalez et al. 2017; Goncalves and Romano, 2017). Furthermore, studies have also reported that rutin could preserve the intact functional β-cells and protect them from further deterioration which is necessary for insulin production (Arora et al., 2021). The pancreas protective effect of PF observed in this study was due to presence of flavonoids in the PF (De la Garza et al. 2014). Also, flavonoids are reported to exert hypolipidemic effect by suppressing HMG–CoA (hexamethyl glucose–coenzyme A) as reported in earlier study (De et al. 2019). It can be said that due to the presence of flavonoids in the PF extract, the PF exhibited glucose and lipid-regulatory potential and the same was confirmed by oral starch and sucrose tolerance test, oral lipid tolerance test, and anti-diabetic and anti-hyperlipidemic assays in DIO mice.

Conclusion

The current study highlights the anti-diabetic and anti-hyperlipidemic potential of methanolic extract of a developed and optimized PF in the DIO mice model for the first time. The study demonstrated that the PF is an effective anti-diabetic agent with multiple therapeutic effects including improved glucose tolerance and TG levels, while preventing metabolic syndrome and lifestyle-related diseases caused by excess consumption of HFD. The PF improved the histological architecture of the pancreatic islets, liver and kidney. These therapeutic activities of PF could be due to the presence of flavonoids as revealed by HPTLC. These results may provide a mechanistic basis for the use of PF as a potent natural functional food, drug or add-on therapy for achieving proper control on obesity-associated diabetes and preventing or delaying diabetic complications. Multi-component synergism is suggested to be responsible for the observed therapeutic effects. However, further studies need to be carried out to explore the mechanism of action of the

individual active phytochemical present in each seed extract along with their combinatorial interactions.

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Author's contributions Tanisha performed the collection of the samples, extraction, designing and performing the experiment. SV guided the first author in performing animal study. MM designed the concept, corrections and drafting of the manuscript. The authors have read and approved this manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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