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Non-polar metabolites of green beans (*Phaseolus vulgaris* L.) potentiate the antidiabetic activity of mesenchymal stem cells in streptozotocin-induced diabetes in rats

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Abstract

Green beans (*Phaseolus vulgaris* L.) are consumed as pods or mature seeds (common beans). The pods were extracted with 95% ethanol and processed to prepare non-polar and polar fractions. Comparing the antihyperglycemic activity of both fractions, non-polar fraction (NPF, 200 mg kg^{-1} day^{-1}) lowered blood glucose in streptozotocin diabetic rats by 65% compared to 57% for the polar fraction at the same dose. When NPF treatment was combined with injection of mesenchymal stem cells (MSC) a 4.4-fold increase in serum insulin and a 73.6% reduction in blood glucose were observed compared to untreated control. Additionally, a significant decrease in malondialdehyde (76.2%), nitric oxide (68.2%), cholesterol (76.1%), and triglycerides (69.5%) and a 1.75-fold increase in HDL concentrations were observed in the group treated with this combination compared to diabetic animals. Interestingly, NPF increased homing of MSC in pancreas potentiating their antidiabetic activity. Finally, 26 compounds were identified in NPF using LC/MS analysis and four were isolated in pure form. The isolated compounds namely calotroproceryl acetate, fridelin, calotroproceryl A, and stigmasterol showed good inhibitory activity against pancreatic lipase with IC$_{50}$ at 1.93, 1.07, 1.34 and 1.44–1 µg/ml, respectively. Additionally, these compounds inhibited α-amylase, albeit at higher concentration, with IC$_{50}$ at 248, 212, 254, and 155 µg/ml for calotroproceryl acetate, fridelin, calotroproceryl A, and stigmasterol, respectively. Our results suggest that green beans extract can potentiate effect of MSC in diabetes directly due to its own antidiabetic effect and indirectly by increasing MSC homing in pancreatic tissues.

Practical applications

It has been suggested in this study that green beans can improve hyperglycemia, oxidative balance in diabetes, so green beans can be promoted as a healthy nutrient for diabetic patients. Green beans also can enhance homing and differentiation of mesnchymal stem cells in the pancreas for future stem cell therapy of type I diabetes.

KEYWORDS
calotroproceryl A, diabetes, fridelin, green beans, mesenchymal stem cells

1 | INTRODUCTION

Diabetes mellitus (DM) is one of the world most prevalent diseases as almost 463 million adults suffer from diabetes with about 232 million cases undiagnosed (Saeedi et al., 2019). DM is manifested primarily as an increase in blood glucose levels (hyperglycemia) due to disorders in insulin secretion and/or defects in insulin function (Viladomiu et al., 2016). Type-I DM is categorized as an autoimmune disease resulting from pancreatic β-cells damage by T-cells cutting insulin production (DiMeglio et al., 2018). Meanwhile, type-II DM is developed later in life due to emergence of insulin resistance (Kolb & Martin, 2017).

While type I DM is treated with subcutaneous insulin administration, the use of conventional oral hypoglycemic drugs is recommended for type II DM (Tan et al., 2019). However the high cost and potential side effects limit the use of these therapeutics by many people in developing countries. For hundred years, scientists were interested in exploring blood glucose-lowering agents from plants especially those with documented ethnobotanical use (Abdel-Sattar et al., 2021; Hago et al., 2021). Thus, investigating bioactive plant secondary metabolites is an essential part of the drug discovery efforts for DM. Singhal et al. (2014) reviewed the glucose and lipid-lowering action of various commonly consumed legumes and concluded that several legumes possess antidiabetic potential and consuming legumes in diet is important in preventing cardiovascular and renal complications of DM.

Among the widely consumed legumes, *Phaseolus vulgaris* L. Fabaceae (common beans, green beans, or French beans) is rich in bioactive constituents with beneficial impact on health (Atchibri et al., 2017; Ocho-Anin Atchibri et al., 2010). Aqueous extract of the pericarps of *P. vulgaris* (green beans) was found to improve metabolic regulation of diabetes in rats (Halenova et al., 2019). Researches have also shown that a combination of the freeze-dried seeds (300 mg/kg) and glibenclamide (0.20 g/kg) resulted in an enhanced antihyperglycemic activities (Ocho-Anin Atchibri et al., 2010). Another study concluded that the aqueous extract of *P. vulgaris* seeds reduced blood glucose and restored normal insulin levels in diabetic rats (Atchibri et al., 2017). Additionally, red kidney beans of *P. vulgaris* showed in vitro anti-diabetic activity by inhibiting α-glucosidase enzyme (Lindawati et al., 2021).

Recently, the use of stem cells (mesenchymal stem cells, MSC) has emerged as a potential cure for DM especially with the limited treatment options for type I DM (Domínguez-Bendala et al., 2012). Stem cells can be derived from embryo, placenta, umbilical cord, bone marrow and blood cells (Moreira et al., 2017). The pancreas influences stem cell differentiation into pancreatic progenitor cells to produce fully functional organ. Hence, most stem cell-based differentiation conventions are focused on the generation of mature, single hormone-expressing, glucose-responsive human β-cells (Jørgensen et al., 2007; Murtaugh, 2007). Recent studies have provided evidence for the effect of herbal extract on proliferation and differentiation of MSC (Saud et al., 2019; Udalamaththa et al., 2016).

The aim of this study is to investigate the antidiabetic effect of the *P. vulgaris* and test possible proliferative effect of *P. vulgaris* extract on MSC. The chemical profile for the non-polar and polar fractions is also investigated by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). Moreover, isolation of the major metabolites from the bioactive fraction was achieved and their potential antidiabetic activity was tested in vitro.

2 | MATERIALS AND METHODS

2.1 | Plant material

Phaseolus vulagris L. legumes were collected at Agricultural Research Center, Giza, Egypt and were authenticated by Dr. Reem Samir Hamdy, Faculty of Science, Cairo University, Giza, Egypt. A voucher specimen is deposited at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University (Voucher No.2-11-2018).

2.2 | Drugs and chemicals

Silica gel H (E-Merck) and silica gel 60 for column chromatography (70–230 nm, Fluka, Germany) were used in the chromatographic procedures. Streptozotocin (STZ) for induction of diabetes, α-amylase enzyme, porcine pancreatic lipase, 4-nitrophenyl octanoate (NPC), orilstat, acarbose, Tris-HCl buffer (pH = 8.5) and sodium phosphate buffer were supplied by Sigma-Aldrich (St' Louis, MO, USA). Glibenclamide was provided from community pharmacy under the trade name of Daonil. Kits for determination of glucose (Sigma-Aldrich), cholesterol and HDL (BioChain, San Francisco, CA, USA), triglycerides (XpressBio, Frederick, MD, USA), nitric oxide (Stressgen, Ann Arbor, MI, USA), rat insulin ELISA (Cusabio) and malondialdehyde (MDA) ELISA (LSBio) were used for assay of different biomarkers.

2.3 | Preparation of ethanolic extract and fractions

The green fresh pods were longitudinally cut and dried. After grinding, 700 g of the dried powder of *P. vulgaris* legumes was extracted with 95% ethanol on cold. The ethanolic extract was evaporated under vacuum at 40°C to yield sold residue (75 g). The residue was suspended in distilled water and defatted by methylene chloride to yield 38 g of non-polar fraction (NPF) and 32 g of polar fraction (PF).

2.4 | Metabolite analysis using liquid chromatography coupled with mass spectrometry (LC/MS)

Chromatographic separation was performed on the Acquity HPLC system (Waters) equipped with Xbridge C18 column (2.1 × 50 mm, particle size 3.5 μm; Waters). Injection volume was 10 μl and chromatographic separation was achieved at flow rate of 0.3 ml/min using the following solvents: solvent A: 0.1% formaic acid, solvent B: 5 mM ammonium formate buffer (pH = 8) and solvent C: 100%

acetonitrile. Elution gradient for positive mode was 0–1 min 90%A:10% C, 1–21 min to 10%A :90%C then isocractic elution for 4 min. For negative ionization mode, 0–1 min 90%B:10% C, 1 and 21 min to 10% B:90% C then isocractic between 21 and 25 min. A hybrid quadrupole time of flight mass spectrometer (Triple TOF-Q 5600+) with electrospray ion source was used for MS analysis Data acquisition was carried out using Analyst TF 1.7.1 software.

2.5 | Isolation and purification of the major metabolites from the bioactive non-polar fraction (NPF)

Ten grams of the NPF were chromatographed on vacuum liquid chromatography (VLC). Gradient elution was adopted as follows: *n*-hexane (100%) followed by 5% increments of $CHCl_3$, up to 100% $CHCl_3$, then by $CHCl_3$/EtOAc (5% increments) to a final concentration of 30% EtOAc. Fractions, each of 100 ml, were collected and monitored by TLC to yield 3 collective fractions (Fr.I–Fr.III). Fr.I (0.6 g) was purified on a silica gel column using isocratic elution with *n*-hexane: ethyl acetate (98:2 vol/vol). Fractions (10 ml each) were collected and monitored by TLC, pooled together and further purified on a silica gel CC using gradient of *n*-hexane with 1% EtOAc increments to yield 30 mg of buff powder (**compound 1**). Fraction II (0.1 g) was chromatographed on silica gel column using *n*-hexane: EtOAc (90:10 vol/vol) to yield **compound 2** as white powder (23 mg) and **compound 3** (40 mg), white crystals. Fr-III (2 g) was purified on two successive silica gel columns to yield **compound 4** (17 mg) as white amorphous powder after precipitation with acetone.

2.6 | In vivo antidiabetic study

2.6.1 | Animals

Albino Wistar rats of similar age and body weight 150–200 g were purchased from the Egyptian organization for biological products and vaccines (Cairo, Egypt). They were fed ad libitum on standard laboratory diet (54% starch, 20% sucrose, 10% corn oil, 10.5% casein) and kept in 12 hr light and dark cycles in a temperature of 25–28°C with access to water. All experimental procedures were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publication No. 85-23, revised 2011) and were approved by the Ethics Committee, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA) PG8/EC8/2018PD.

2.6.2 | Induction of diabetes

Before induction of diabetes, animals were fasted for 12 hr. Diabetes was induced by single intraperitoneal injection of streptozotocin dissolved in cold freshly prepared 0.1 M citrate buffer (pH 4.5) at dose of 50 mg /kg. After induction, rats were allowed to administer 5% glucose solution to avoid hypoglycemia induced by STZ. Diabetes was confirmed by the presence of polyuria and blood glucose level above 300 mg/dl after 48 hr of induction (Kasali Felicien et al., 2016).

2.6.3 | Experimental design

To determine acute toxicity of the extracts, a single dose of 500 mg/kg polar and non-polar fractions were administered to different groups of rats. The mortality rate was observed, and no mortality was recorded for a 24-hr period. Two doses of 100 and 200 mg/kg were chosen for further investigation based on a previous study done on *P. vulgaris* pods (Venkateswaran & Pari, 2002).

To determine the fraction and the dose that to be used throughout the study, 36 diabetic rats were randomly divided to six study groups. Group 1: received a daily oral dose of *P. vulgaris* PF (polar fraction) 100 mg/kg, group 2: received daily oral PF 200 mg/kg, group 3: received a daily oral *P. vulgaris* NPF (non-polar fraction) 100 mg/kg, group 4: received a daily oral NPF 200 mg/kg, group 5 was treated by daily oral glibenclamide (0.6 mg / kg) and group 6: diabetic control group received vehicle only. All doses were administered through an orogastric tube for 30 days.

Based on blood glucose levels observed at the end of the study period, the dose NPF 200 mg/kg was selected for further investigation.

For testing the proliferative effect of NPF on MSC, 36 rats were randomly divided into six groups (six rats each). The experiment duration was 30 days and daily doses were delivered through orogastric tube. Animals were assigned as: group 1: normal control that received only normal saline (10 ml/kg) while diabetic rats were randomly divided to the study groups 2 to 6 as follows: group 2: diabetic control group received vehicle only, group 3: received daily dose of glibenclamide (0.6 mg/kg), group 4: received a daily dose of *P. vulgaris* NPF 200 mg/kg, group 5: received stem cells in a single intraperitoneal injection of 1×10^6 cells per animal and group 6: received both stem cells (1×10^6 cells/rat) and daily dose of *P. vulgaris* (NPF 200 mg/kg).

After 30 days; the rats were fasted overnight and deeply anesthetized. Blood samples were collected from inferior vena cava into Eppendorf tubes without addition of EDTA using capillary tubes. Dissection was done to isolate the liver and pancreas.

2.6.4 | Sample preparation

The collected blood samples were centrifuged at 1000 rpm for 10 min, serum was separated and stored at −20°C for determination of glucose, insulin, cholesterol, HDL, triglycerides and nitric oxide. Sacrification was conducted via humane killing (cervical dislocation) and dead bodies were discarded by incineration. The

pancreas was excised immediately and rinsed with isotonic saline. Pancreas was suspended in 10% formalin and stored at temperature of −20°C to undergo radioimmunoassay using fluorescent microscope. Additionally, pancreatic tissue was minced and a homogenate was prepared with 10% (wt/vol) phosphate-buffered saline (PBS, 0.1 mol/L, pH = 7.4). The homogenate was centrifuged at 10,000 rpm for 5 min at −4°C. The supernatant was directly used for the determination of pancreatic MDA.

2.6.5 | Measurement of biochemical parameters

Pancreatic MDA was assayed by ELISA using TECAN Spectra Classic plate reader (Germany). Serum glucose, cholesterol, triglycerides and HDL were all measured spectrophotometrically using Beckman and Coulter AU480 chemical analyzer while serum insulin was determined by ELISA using TECAN Spectra Classic plate reader (Germany).

2.6.6 | Isolation and propagation of bone marrow-derived mesenchymal stem cells from rats

The extraction of bone marrow was done by the flush of the tibiae and femurs of six-week-old white albino male rats with Dulbecco's modified Eagles medium supplemented with 10% bovine fetal serum. A density gradient was used to isolate nucleated cells and re-suspend them in 1% penicillin streptomycin culture medium. Cells were incubated at temperature 37°C and CO_2 at 5% for 12–14 days as primary culture until the production of large colonies. After the formation of large colonies, they were washed with phosphate buffer saline twice and the cells were trypsinized using 0.25% trypsin dissolved in 1 ml EDTA at temperature 37°C for 5 min. Cells were centrifuged, re-suspended in a serum-supplemented medium and incubated which represented the first passing culture (Alhadlaq & Mao, 2004). On day 14, cell colonies were counted after being trypsinized. The identification of mesenchymal stem cells was done using their morphology, power to differentiate and their adherence (Jaiswal et al., 1997). Undifferentiated mesenchymal stem cells were labeled with PKH26 according to the recommendations of the manufacturer (Sigma, Saint Louis, Missouri, USA).

2.7 | Pancreatic lipase inhibition assay

Inhibition of pancreatic lipase was measured using the assay described by Conforti et al. (2012). Briefly, the reaction mixture was: 100 μl of 5 mmol/L NPC, 4 ml of Tris-HCl buffer (pH = 8.5), 100 μl of test compound (12 to 100 μg/ml) and 100 μl of enzyme solution (1 mg/ml). The mixture was incubated at 37°C for 25 min before the substrate was added. In the control, the compound was replaced with the same volume of DMSO. The absorbance was measured in cuvettes at 412 nm using ELX 808 (BioTek Instrumental, Italy).

A blank sample without the enzyme was measured for each compound. For comparison, orlistat was tested at a final concentration of 20 μg/ml.

Percentage of inhibition (I%) was calculated by using the following formula:

$$I\% = 100 - \left[\{A\,sample/A\,control\} \times 100 \right]$$

where A represent absorbance at 412 nm.

2.8 | α-Amylase inhibition assay

The α-amylase inhibition assay was performed as stated by Sut et al. (2020) with some modifications. Starch solution (0.5% wt/vol) was used as a substrate (potato starch in 20 mmol/L sodium phosphate buffer with 6.7 mmol/L sodium chloride) and α-amylase enzyme solution (1 mg/ml in cold distilled water). Tested compounds were individually dissolved in phosphate buffer (75 to 300 μg/ml). The colorimetric reagent was prepared by mixing sodium potassium tartrate solution (12.0 g/ 8.0 ml of 2 M NaOH) and 96 mmol/L of 3,5-dinitrosalicylic acid solution. Samples were mixed with starch solution and left to react with α-amylase solution in alkaline conditions at 25°C. The generation of maltose was quantified by the reduction of 3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylicacid. Acarbose was used as positive control. Absorbance (A) was measured at λ_{max} 540 nm using ELX 808 (Bio Tek Instrumental, Italy). Percentage of inhibition (I%) was calculated by using the following formula:

$$I\% = 100 - \left[\{A\,sample/A\,control\} \times 100 \right].$$

2.9 | Statistical analysis

All the assays were carried out in triplicate. The results were expressed as mean values and standard deviations (SDs). The IC_{50} (concentration necessary for 50% inhibition of enzyme activity) was calculated by constructing a linear regression curve plotted using concentrations and I% for α-amylase and pancreatic lipase. Comparison between 2 groups was done by unpaired student t-test and $p < .05$ was considered statistically significant. All analyses were done using the SPSS v22.0 (IBM, Chicago, USA) and Graphpad Prism (6.01) (California, USA).

3 | RESULTS AND DISCUSSION

3.1 | Identification of the polar and non-polar fractions via HPLC-qTOF MS/MS

UPLC/MS analysis allowed a comprehensive analysis of the metabolites profile of both polar (PF) and nonpolar fraction (NPF) of *P. vulgaris* using high resolution mass and fragmentation pattern in comparison with available literature. As presented in Figure 1 and

Tables S1 and S2, a total of 50 compounds were detected in the positive mode, of which 48 were identified.

3.1.1 | Secondary metabolites of the polar fraction

Twenty-five flavonoids including flavones, flavanols, isoflavones, and flavans were identified in the PF, Table S1 and Figure 1, of which flavones were represented by ten peaks; **1**, **3**, **5**, **6**, **9**, **10**, **13**, **14**, **18** & **23**, Figure 1a. Peak (**1**), eluted at retention time (R_t) 1.83 min, showed an m/z for $[M + H]^+$ at 611.1608 which corresponds to molecular formula $C_{27}H_{30}O_{16}$. In MS/MS mode, fragments at m/z 449 (M + H-162), and 287 (M + H-162–162) were observed due to consecutive loss of hexose. Therefore, peak 1 was tentatively identified as luteolin-dihexoside. Similarly, peak **14** at $R_t = 11.98$ min was identified as luteolin-8-C- hexoside (orientin) by the characteristic fragments 359 (M + H-90) corresponding to C-8 glycoside, and fragment at m/z 287 for luteolin aglycone. Likewise, peak **10** was identified as (iso) orientin, as suggested by the fragment at m/z 329 corresponding to [(M + H 449.10)-120] indicative

of C-6-glycoside (Silva Mathias & Rodrigues de Oliveira, 2019). Apigenin was also detected as sugar conjugates at peaks **3**, **5** & **18**; including its O-glycoside, apigenin 7-O hexoside (peak **18**) which was easily differentiated from the C-glycosides observed in peaks: **3**, **5**, **9** by the loss of hexose moiety (–162) (Mekky et al., 2020). Similarly, peak **9**, a C-glycoside, was identified as vitexin due to the characteristic loss of 90 amu observed for C-8 glycoside (Geng et al., 2016). On the other hand, peak **5** with molecular ion at m/z 565.1562 corresponding to $C_{21}H_{18}O_{13}$ was identified as apigenin di-C-hexoside (vicenin II) denoted by fragments produced by neutral loss of 120 amu for C-6 glycoside and that produced by neutral loss 90 amu for C-8 glycoside (Farag et al., 2016; Geng et al., 2016). Additionally, apigenin-pentosyl-hexoside (peak **3**) was identified by the loss of hexose (–162) followed by loss of pentose (–132) to give the aglycone molecular ion at m/z 271. Two other compounds (peaks **6** & **13**) were identified as isomers of acacetin-rhamnosyl-hexoside in peak **13**, with m/z 593.23 for $[M + H]^+$ and fragments at 447 for [M + H-146] equivalent to the loss of rhamnosyl moiety and at m/z 285 for acacetin. Similarly peak **6** showed the same pattern of fragmentation, Table S1.
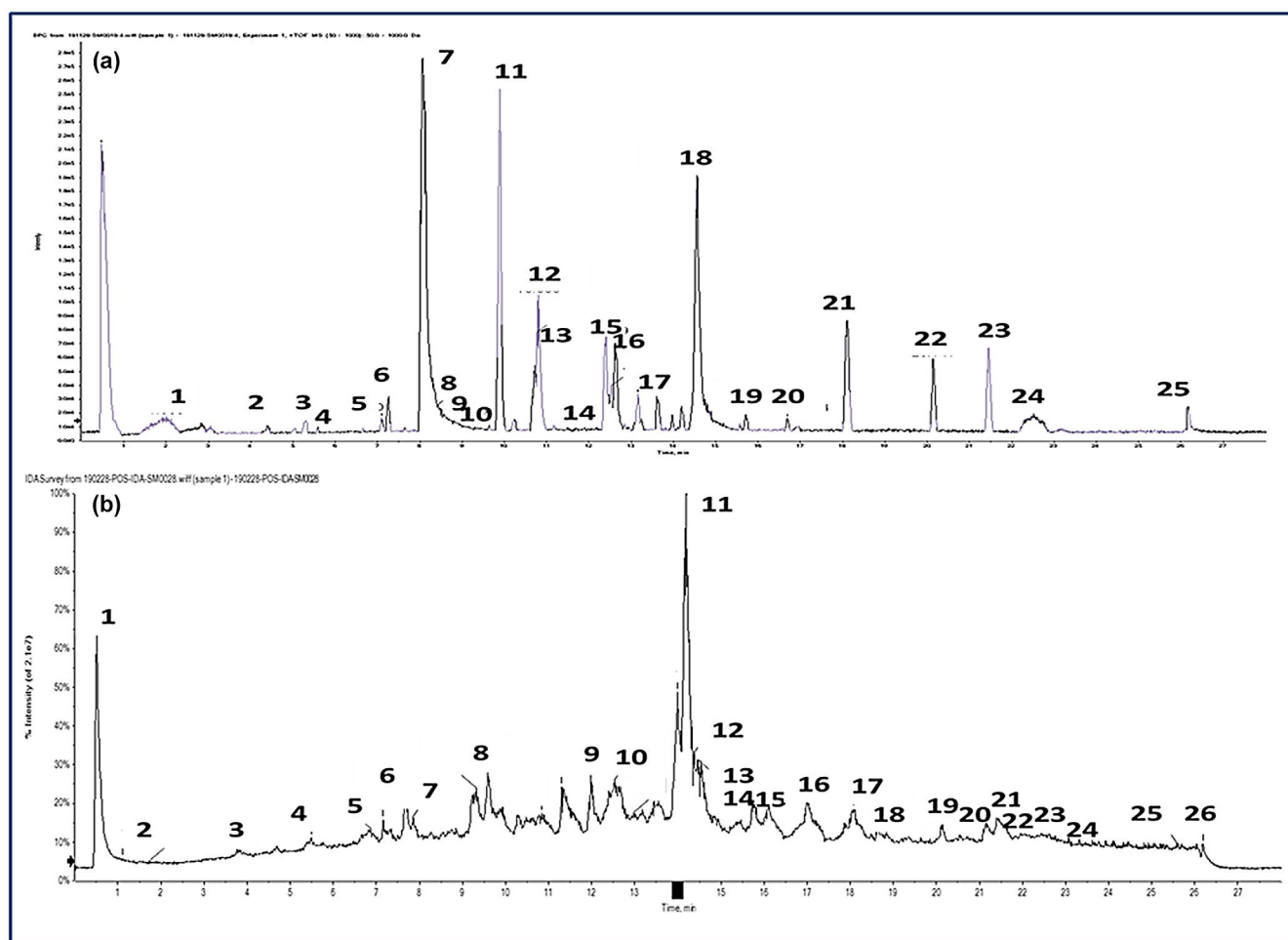
FIGURE 1 HPLC/MS chromatogram of *Phaselous vulgaris* extract HPLC/MS chromatogram in positive ionization mode of *P. vulgaris* (a) polar fraction and (b) non polar fraction. Peak numbers correspond to compounds identified and listed in Table S1 for chromatogram A and those listed in Table S2 for chromatogram B

Flavonols were represented by 13 peaks, nine of which were quercetin conjugates representing the most abundant metabolites, in addition to three kaempferol and one rhamnetin glycoside. Peaks **2, 4, 7, 12, 16, 17, 20, 22** & **23** showed a fragment ion at m/z 303.05 indicating quercetin aglycone (Chen et al., 2015). Peaks **16, 20** & **23** were characterized as quercetin-rhamnoside due to the loss of a rhamnosyl moiety (−146 amu). Likewise, peaks **7, 17** & **22** were annotated as quercetin-O-hexoside as revealed by the loss of hexosyl moiety (−162 amu) for glucose/galactose (Farag et al., 2016). Meanwhile, peaks **2** & **12** revealed the loss of rhamnosyl moiety (−146 amu) followed by the loss of a hexosyl moiety (−162) from the molecular ion $[M + H]^+$ at m/z 611 amu to finally give m/z 303.10 as a base peak and were assigned as rutin isomers (Mekky et al., 2020). Finally, peak **4** displayed a molecular ion peak at m/z 479 that was annotated as quercetin glucuronoide, as suggested by the loss of 176 amu consistent with a glucuronoyl moiety (Farag et al., 2016). As for peaks **11, 15** & **25**, their fragmentation pattern allowed their identification as kaempferol–rhamnoside, kaempferol–hexoside and kaempferol, respectively (Chen et al., 2015). The last identified flavone peak (**18**) exhibited a molecular ion at m/z 479.1193 for ($C_{22}H_{22}O_{12}$) which yielded a fragment ion at m/z 317 ascribable to (*iso*)rhamnetin aglycone with a loss of 162 amu (a hexose moiety), and was tentatively identified as (*iso*) rhamnetin-O-hexoside. This is in addition to, an isoflavone glycoside (peak **8**) displaying an $[M + H]^+$ at m/z 433.1134 and fragment ions at m/z 271.08 and 227.09, 198.33,which are typical for genistein-hexoside (Zhao et al., 2018). The last identified peak (**21**), showed a fragmentation pattern consistent with naringenin hexoside as inferred from the fragment ion at m/z 273.08 [M + H-162] that correspond to naringenin aglycone (Chen et al., 2015).

3.1.2 | Identification of secondary metabolites in the non-polar fraction

A total of 26 compounds were detected in the LC/MS chromatogram of the non-polar fraction, where 23 compounds were identified, Figure 1b and Table S2. Flavonoids with different classes represented the majority of identified compound and were mainly aglycones or mono-glycosides. Additionally, several hydroxyl cinnamic acid derivatives and sterol/triterpenoids were annotated in the non-polar fraction.

Hydroxycinnamic acid conjugates
These were represented by five peaks; **5**–**8** & **11**, Table S2 and Figure 1b. Peak **11** showed an m/z at 181 for $[M + H]^+$ with fragment ion at m/z 137 and was annotated as caffeic acid (Seraglio et al., 2016). Two coumaric acid isomers were observed at R_t = 6.88 and 9.28 min.(peaks **6** & **8** respectively) and were identified by the relative intensity of the fragment ion at m/z 119 (M + H-HCO$_2$) as p-coumaric acid & o-coumaric acid respectively (Wang et al., 2012). Peaks **5** & **7** were annotated as sinapic acid and sinapoyl hexoside as

inferred by the ions at m/z 225 for $[M + H]^+$ and 165 corresponding to loss of two methoxy groups (Seraglio et al., 2016).

Flavonoids
In NPF, flavonoids were represented by 12 peaks (Table S2); four glycosides (peaks **3, 4, 9** & **10**) and eight aglycones (peaks **13, 14, 17**–**23**). Peaks **3** & **9** were identified as apigenin-O-hexoside and luteolin-8-C-hexoside as discussed previously. Peak **10** was assigned as gossypetin-O-hexoside with molecular ion peak at m/z 481.1477 equivalent to $C_{21}H_{20}O_{13}$ and a base peak fragment ion at m/z 319 for gossypetin aglycone (Ezzat et al., 2016). The glycoside at peak **3** was tentatively assigned as diadzein (4′-hydroxyisoflavone-7-O-hexoside) based on its $[M + H]^+$ ion at m/z 417.1518 and the fragment ion produced at m/z 255.06 for corresponding to diadzein aglycone (Salama et al., 2015). Analysis of other flavonoidal peaks identified peak **19** as diadzein aglycone and peaks **17, 18, 21** & **22** as acacetin, apigenin, luteolin & quercetin, respectively. Peaks **20** and **22** were annotated as formonetin and myricetin based on their fragmentation pattern (Ezzat et al., 2016). Peaks **13** & **14** were assigned as hesperitin (Brito et al., 2014) and luteoforol (3 3′ 4′ 5 7-pentahydroxyflavan), respectively based on their fragmentation pattern (Lima et al., 2020).

Sterols & triterpenes
These were represented by peaks **24**–**26**. Peak **24** yielded $[M + H]^+$ with m/z 427.3783 and two main fragments at m/z 409.34 [M + H − H$_2$O]$^+$ and 369.31 [M + H − C$_2$O]$^+$, in agreement with friedelin (Naumoska & Vovk, 2015). Similarly, peak **26** showed a molecular ion peak $[M + H]^+$ at m/z 427.7670 and MS^2 fragmentation characteristic for taraxasterol (Avula et al., 2011; Zhang et al., 2015). Peak **25** was identified as a sterol from its molecular ion at m/z 412.3705 corresponding to the formula $C_{29}H_{48}O$ and revealed a base peak fragment at m/z 395, and, was identified as stigmasterol (Naumoska & Vovk, 2015).

Nitrogenous compounds
These were represented by peaks **2** & **15**. Peak **2** revealed $[M + H]^+$ at m/z 163.126, followed by product ions 144.07 (−H$_2$O) and 126.05(−2H$_2$O), then a very distinguished peak at m/z 103.03 conforming the loss of N(CH$_3$)$_2$. This fragmentation pattern is in accordance with that of Carnitine (Sowell et al., 2011). Peak **15** was identified as caffeine from its MS^2 fragments (Mendes et al., 2019).

3.2 | Antihyperglycemic effects of the polar and non-polar fractions on diabetic rats

The non-polar fraction of *P. vulgaris* pods (NPF) was more potent than the polar fraction (PF) in reducing serum blood glucose at both 100 and 200 mg/kg dose, Table 1. Therefore, the NPF was chosen for further study on its synergistic effect in combination with MSC at 200 mg/kg.

TABLE 1 The concentration of serum glucose in animals treated with different doses of the polar and nonpolar fraction

Study group	Serum glucose (mg/dl)
PF (100 mg kg^{-1} day^{-1})	180.5 ± 5.6[a–d]
PF (200 mg kg^{-1} day^{-1})	163.5 ± 5.6[abd]
NPF (100 mg kg^{-1} day^{-1})	143.8 ± 5.6[a–d]
NPF (200 mg kg^{-1} day^{-1})	130.2 ± 2.6[a–c]
Glibenclamide	88.00 ± 6.4[b–d]
Diabetic control	379.8 ± 30.5[acd]

Note: Results are expressed as mean ± SD. [a]Significant difference from diabetic control. [b]Significant difference from glibenclamide group. [c]Significant difference from NPF 100 mg/kg group. [d]Significant difference from NPF 200 mg/kg group.

Abbreviations: NPF, non-polar fraction of *Phaseolus vulgaris* pods; PF, polar fraction of *Phaseolus vulgaris* pods.

3.3 | The effect of different treatment on glucose and insulin levels

Diabetes was successfully induced by single intraperitoneal injection of STZ (50 mg/kg) and was accompanied by hyperglycemia and reduction of serum insulin level, Figure 2. These results are in agreement with Hegazi et al. (2021). A significant reduction in blood glucose was observed in rats treated 200 mg/kg of NPF (65.6%) or injected with a single dose of mesenchymal stem cells (65.4%), $p < .0001$, when compared to diabetic control group, Figure 2a. A greater reduction (73.6%) was observed in animals that received a combination of NPF and, $p < .0001$. Furthermore, no significant difference was observed between the group treated via standard drug glibenclamide and that treated with combination therapy.

Similarly, a significant elevation of serum insulin concentration was observed in groups treated with NPF, stem cells and combination of both compared to diabetic control group ($p < .0001$). Insulin levels in rats treated with 200 mg kg^{-1} day^{-1} of NPF or stem cells were estimated at 5.1 and 5.4 ± 0.2 mIU/mL compared to a concentration of 1.3 ± 0.1 mIU/ml in untreated diabetic rats. Meanwhile, serum insulin concentration in the group that received glibenclamide treatment was reported at 5.2 ± 0.2 mIU/ml which was comparable to NPF and stem cell treatment $p = .229$ and 0.053, respectively. Finally, stem cells and NPF combination group increased the serum insulin level (5.7 ± 0.2) more than glibenclamide in a significant manner ($p = .0015$), Figure 2b. This result suggests a significant potentiation of insulin secretion from the pancreatic β cells and an increased sensitivity of β-cells to generate insulin by both NPF and MSC. It was suggested previously that mesenchymal cells can differentiate to islets beta cells (Chen, 2004) which can explain the elevation of insulin levels and consequently the decrease in glucose levels.

3.4 | The effect of different treatment on MDA and NO levels

Oxidative stress is one of the main factors behind morbidity associated with DM complications (Mylona-Karayanni et al., 2006).

Induction of diabetes with STZ in rats increases oxidative stress in the pancreas triggering its damage (Lenzen, 2008). Malondialdehyde (MDA) is produced from the peroxidation of polyunsaturated fatty acid and was measured in the current study as an indicator for oxidative stress (Pizzino et al., 2017).

Diabetic control group showed a significant elevation in the pancreatic MDA level (25.2 ± 0.6 ng/g tissue) when compared to all other study groups ($p < .0001$). Meanwhile, animals treated with NPF showed 80% decrease in pancreatic MDA when compared with diabetic group, $p < .0001$. Based on LC/MS analysis, NPF was enriched with powerful antioxidants including flavonoids and phenolic acids. Our findings are also supported by a previous report of the strong antioxidant effect of the aqueous extract of *P. vulgaris* pods (Kyznetsova et al., 2015). On the other hand, MSC treated group showed lower antioxidant activity which was probably of secondary origin due to reduced hyperglycemia. Combination of NPF and stem cells provided a significantly higher antioxidant activity than MSC alone but was significantly less than NPF alone.

Similarly, a significant increase in serum nitric oxide (NO) level was observed in the diabetic control group compared to all other groups ($p < .0001$). Animals treated with either NPF or MSC showed lower NO level than diabetic control group, but its level remained higher than normal control group ($p < .0001$). Meanwhile, MSC + NPF combination showed the lowest NO level (7.4 ± 0.1 μmol/L) compared to other study groups (Figure 2d). Several studies have reported higher level of NO in diabetic patients (Adela et al., 2015; Mylona-Karayanni et al., 2006), while others have demonstrated a decrease in nitric oxide levels in diabetes (Apakkan Aksun et al., 2003). This discrepancy is due to the fact that NO plays complex roles ranging from maintaining the endothelial function to acting as an inflammatory mediator (Hegazi et al., 2021). The current study revealed that animals treated with NPF + MSC showed reduced NO levels (below normal control group). MSC were previously reported to have an anti-inflammatory action that attenuates peripheral neuropathy in diabetes (Waterman et al., 2012).

3.5 | The effect of different treatment on serum lipids

Dyslipidemia is commonly reported in STZ-induced diabetic rats where an increase in TGs, cholesterol and decrease in HDL are expected (Nandini & Naik, 2019; Oskouei et al., 2019). Similar effects were shown in diabetic untreated rats used in this study, Figure 3. All treatment groups showed a significant reduction in serum cholesterol level compared to diabetic control group ($p < .0001$; Figure 3a) where the cholesterol lowering effect was more pronounced in group treated with a combination of NPF and MSC compared to NPF treated group ($p = .0096$). Moreover, the serum cholesterol level in the combination group was comparable to the normal controls ($p = .2614$) and glibenclamide treated group ($p = .2162$).

(a)

(b)

(c)

(d)
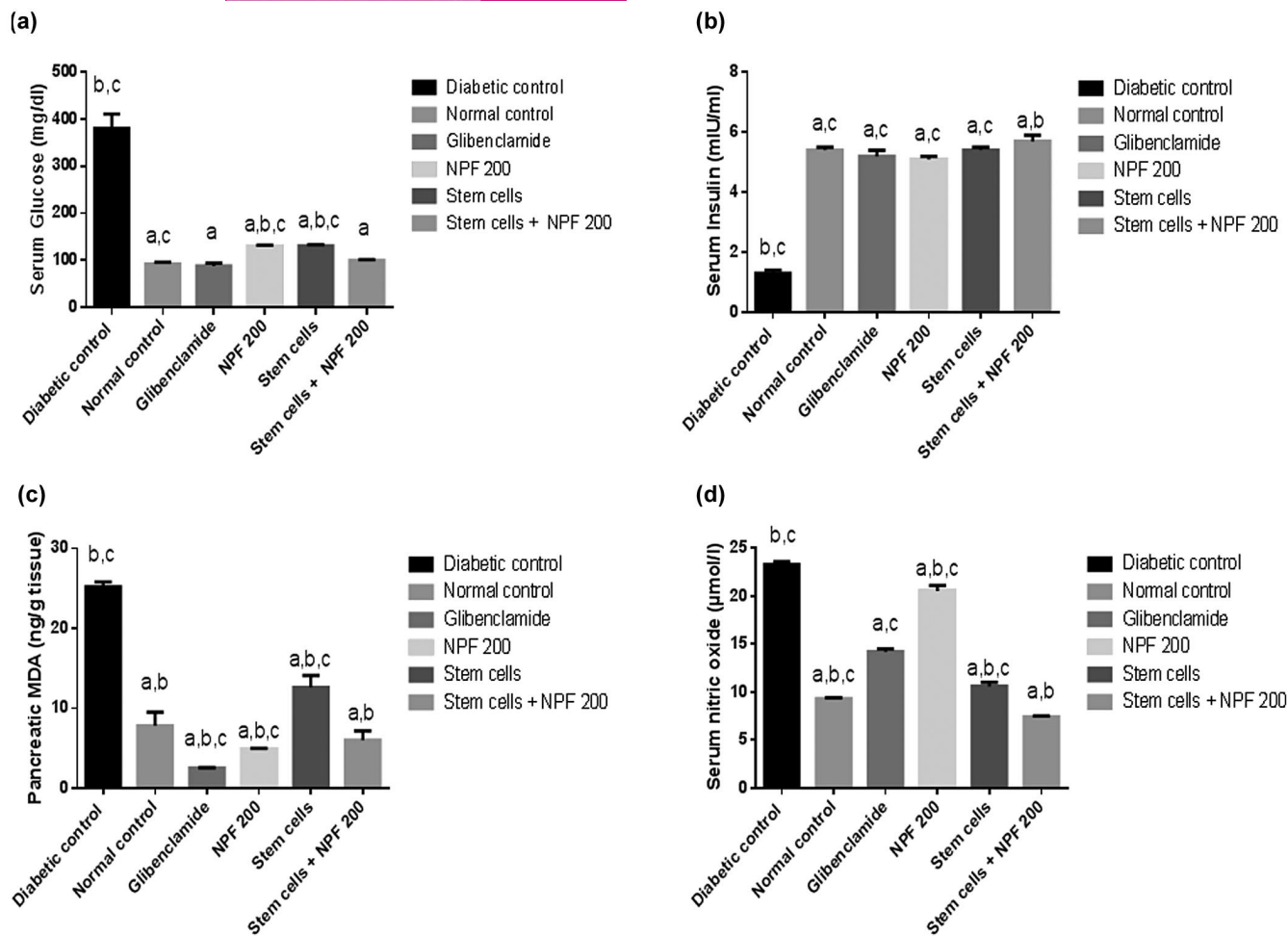
FIGURE 2   The concentration of different serum biomarkers measured in different treatment groups. Serum levels of (a) glucose (mg/dl), (b) insulin (mIU/ml), (c) pancreatic malondialdehyde (ng/g tissue), and (d): serum nitric oxide (µmol/L) in different study groups. Results are expressed as mean ± *SD*. NPF, non-polar fraction. a: Significant difference from diabetic control, b: Significant difference from glibenclamide group, c: Significant difference from stem cells + NPF *P. vulgaris* 200 mg/g group

(a)

(b)

(c)
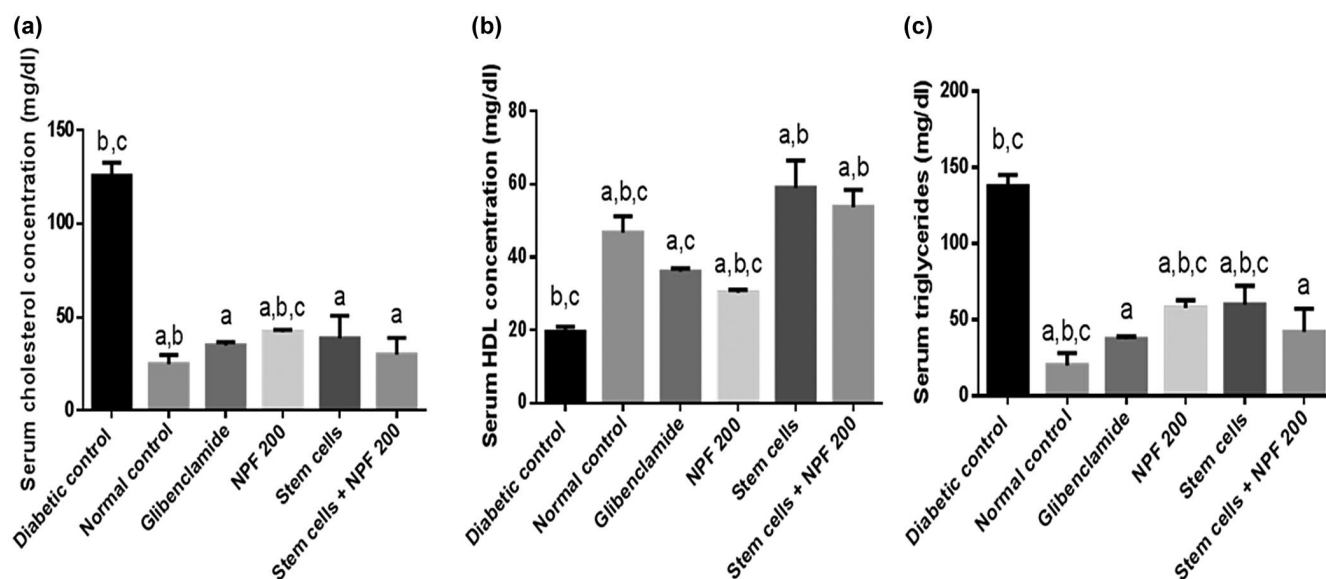
FIGURE 3   The lipid profile in different study groups. Parameters measured are (a) serum cholesterol, (b) serum HDL and (c) serum triglycerides) in mg/dl. Results are expressed as mean ± *SD*. NPF, non-polar fraction. a: Significant difference from diabetic control, b: Significant difference from glibenclamide group, c: Significant difference from stem cells + NPF *P. vulgaris* 200 mg/g group

NPF, MSCs and their combination caused significant increase in HDL compared to diabetic control group ($p < .0001$), Figure 3b. A two-fold increase in HDL levels was observed in the group treated with MSC and MSC + NPF combination compared to animals treated with NPF only ($p < .0001$). Meanwhile, triglycerides levels in the group treated with a combination of MSC and NPF were significantly less than in animals treated with either MSC or NPF alone and similar to that induced by glibenclamide, Figure 3c.

3.6 | Effect of NPF on homing of mesenchymal stem cells in the pancreas

Pancreatic tissue showed significant immunofluorescence due to PKH26 labeled MSC in animals treated with MSC, Figure 4a. However, higher immunofluorescence was detected in pancreas tissues of animals receiving a combination of NPF and MSC as shown in Figure 4b suggesting that NPF of *P. vulgaris* extract increased homing of the injected MSC in pancreas thus enhancing antidiabetic effect of MSC if used alone.

3.7 | Identification of the isolated compounds

Four compounds were isolated from the bioactive NPF of *P. vulgaris* pods. By comparing their ^{1}HNMR and ^{13}CNMR (Table 2) with published data, these compounds were identified as Calotroproceryl acetate A (ursa-5,12,20(30)-trien-18α-H-3β-yl acetate, **1**) (Ibrahim et al., 2012), Friedelin (3-oxo-Friedelane, **2**) (Farozi et al., 2015), Calotroprocerol A (ursa-5,12,20(30)-trien-18α-H-3β-ol, **3**) (Ibrahim

et al., 2012), and stigmasterol (**4**) (Kumar et al., 2013). Structures of the isolated compounds are shown in Figure 5.

3.8 | In vitro antidiabetic assay for the isolated compounds

Pancreatic lipase is the key enzyme in lipid digestion (Gondokesumo et al., 2017). Since diabetes is accompanied by excessive accumulation of lipids in the pancreas, inhibition of pancreatic lipase is a crucial step in diabetes control. This inhibition could protect the pancreas by decreasing lipid absorption thus retaining insulin production from β-cells (Yang et al., 2014; You et al., 2011). The major isolated compounds from NPF showed strong lipase inhibitory activity as depicted in Table 3. The results revealed that at concentration of 100 μg/ml, 89%, 95%, 94% and 93% inhibition of the lipase activity were observed for calotroproceryl acetate A (**1**), friedelin (**2**), calotroprocerol A (**3**), and stigmasterol (**4**), respectively by 89%, 95%, 94%, & 93% which is similar to results obtained with standard orlistat (92%).
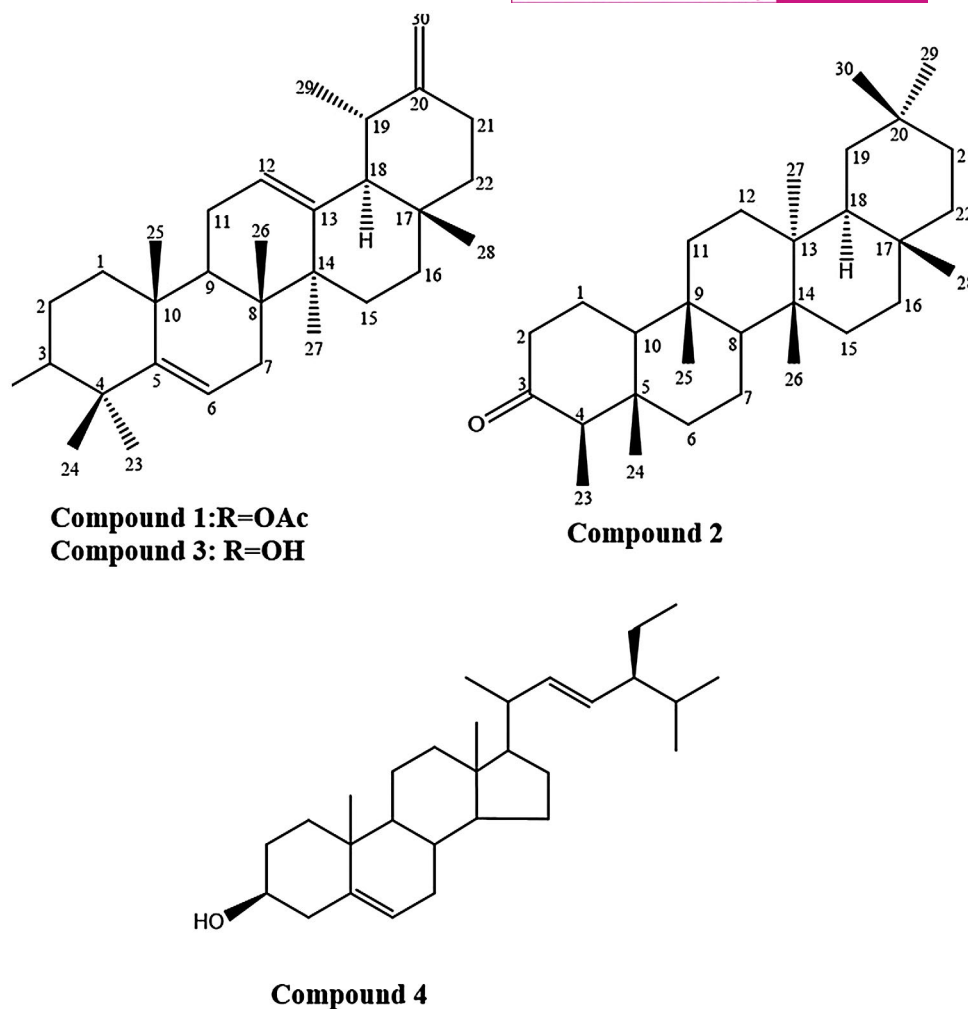
Additionally, significant α-amylase inhibitory activity was observed for the isolated compounds almost the same as the standard acarbose. Inhibition of α-amylase, a key enzyme in carbohydrate digestion, plays an important role in the management of DM by preventing release of glucose from dietary starch (Martinez-Gonzalez et al., 2019). In support to our findings, friedelin has been previously reported to exert antidiabetic activity by inhibiting α-glucosidase and α-amylase enzymes (Atta-ur-Rahman et al., 2008; Rodrigues et al., 2017). Similarly, it was reported that stigmasterol exerts antidiabetic activity by inhibition of both α-glucosidase and α-amylase at
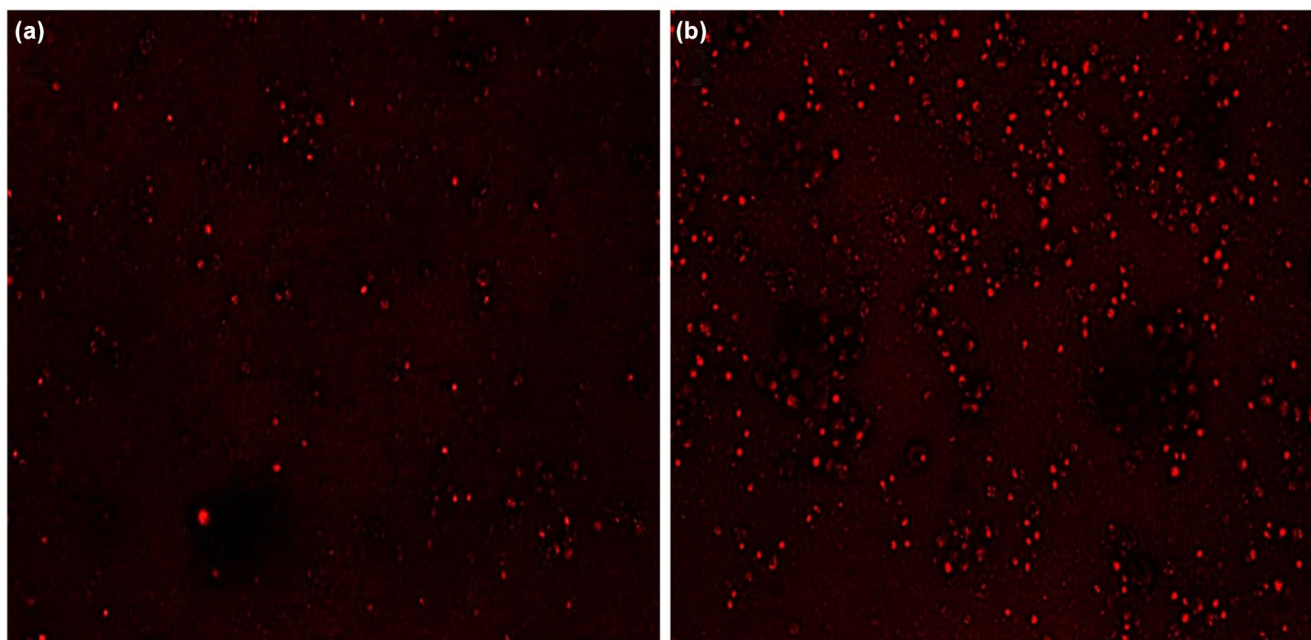
FIGURE 4 Effect of non-polar fraction on homing of mesenchymal stem cells in the pancreas. (a) The immunofluorescence of the labeled mesenchymal stem cells alone, (b) high concentration of immunofluorescence when the labeled mesenchymal stem cells + NPF

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TABLE 2 ^{1}HNMR and ^{13}CNMR data of the isolated compound

C No.	Compound 1 δH ppm	Compound 1 δc ppm	Compound 2 δH ppm	Compound 2 δc ppm	Compound 3 δH ppm	Compound 3 δc ppm	Compound 4 δH ppm	Compound 4 δc ppm
1	1.68,1.11 (2H,m)	38.5	1.5, 1.9 (1H, m)	22.2	1.62,1.0 (2H,m)	38.8	2.22 (m), 2.98 (d, J = 12.8 Hz)	37.2
2	2.02,1.97 (2H,m)	28.0	2.22 (1H, m); 2.31 (1H, m)	41.5	2.38,2.28 (2H,m)	28.7	1.78 (m), 2.14 (m)	31.5
3	3.96 (1H,dd, J = 5.3;4.1)	80.5	–	213.2	2.96 (1H, dd, J = 5.3;4.1)	79.0	3.45 (1H,m)	71.8
4		37.7	2.25 (2H,m)	58.2		41.7	1.93 (2H,m)	42.2
5		145.2		42.1		145.1		140.7
6	5.06 (1H, br.s)	121.6	1.74,1.28 (2H,m)	41.2	5.05 (1H,br.s)	121.7	5.29 (1H,br.s)	121.7
7	1.58,1.34 (2H,m)	33.3	1.49,1.36 (2H,m)	18.2	1.48,1.24 (2H,m)	32.9	1.81 (2H,m)	31.9
8		40.0	1.38 (1H,m)	53.1		40.0		31.9
9	1.58 (1H,m)	47.6		37.4	1.45 (1H,m)	47.7	1.42 (1H,m)	50.1
10		37.4	1.53 (1H,m)	59.4		36.8		36.5
11	1.92,1.65 (2H,m)	23.6	1.45,1.26 (2H,m)	35.6	1.83 (2H,m)	23.3	1.66 (2H,m)	21.0
12	5.10 (1H, br.s)	124.3	1.33,1.36 (2H,m)	30.5	5.11 (1H,br.s)	124.4		39.6
13		139.6		39.7		139.5		42.3
14		42.0		38.3		41.7		56.7
15	1.63, 1.13 (2H,m)	27.9	1.47,1.27 (2H,m)	32.4	1.53, 1.03 (2H,m)	27.2	1.58 (2H,m)	24.3
16	1.90,1.63 (2H,m)	23.6	1.58,1.35 (2H,m)	36.0	1.83, 1.43 (2H,m)	23.5	1.79 (2H,m)	28.2
17		34.8		30		33.7		56.0
18	1.92(1H,d, J = 6.9)	47.2	1.56 (1H,m)	42.8	1.92 (1H,d, J = 6.9)	48.3	0.93 (3H,s)	11.9
19		41.7		35.3		41.5	0.99 (3H,s)	19.3
20		151.0		28.1		150.4		36.1
21	2.30,2.21 (2H,m)	32.8	1.50,1.31 (2H,m)	32.7	2.11, 2.01 (2H,m)	31.2	0.79 (3H, d, J = 6.3 Hz)	18.9
22	1.31, 0.92 (2H,m)	39.8	1.51,0.95 (2H,m)	39.2	1.33, 1.04 (2H,m)	39.6	4.91 (1H, dd, J = 8.5, 15.0)	138.3
23	0.99 (3H,s)	29.1	0.93 (3H,d, J = 6.4 Hz)	6.8	0.94 (3H,s)	29.7	5.05 (1H, dd, J = 8.5, 15.0)	129.2
24	0.80 (3H,s)	15.7	0.65 (3H,s)	14.6	0.80 (3H,s)	15.6	–	45.8
25	0.95 (3H,s)	16.8	0.80 (3H,s)	17.9	0.92 (3H,s)	16.1	–	29.3
26	0.89 (3H,s)	16.8	0.81 (3H,s)	20.2	0.89 (3H,s)	16.8	0.60 (3H, d, J = 6.7 Hz)	19.0
27	1.06 (3H,s)	26.6	0.88 (3H,s)	18.6	0.96 (3H,s)	26.6	0.71 (3H, d, J = 6.7 Hz)	19.8
28	0.72 (3H,s)	17.5	0.98 (3H,s)	32.0	0.72 (3H,s)	17.4	–	23.0
29	0.94 (3H,d, J = 6.3)	18.2	1.11 (3H,s)	35.0	0.86 (3H,d, J = 6.3)	18.0	0.77 (3H, t, J = 6.7 Hz)	11.9
30	4.50 (1H,br.s) 4.38 (1H,br.s)	107.1	1.18 (3H,s)	31.9	4.61 (1H,br.s) 4.49 (1H,br.s)	109.3		
31		173.6						
32		21.4	2.19 (3H,s)					

FIGURE 5 Structures of major compounds isolated from non polar fraction of green beans

TABLE 3 The inhibitory activity of the isolated compounds from NPF on, pancreatic lipase and α-amylase

Tested sample	Pancreatic lipase		α-Amylase	
	IC_{50} (µg/ml)	% inhibition at 100 µg/ml	IC_{50} (µg/ml)	% inhibition at 300 µg/ml
1	1.93 ± 1.2	89 ± 4.1	248.4 ± 3.8	64 ± 4.3
2	1.07 ± 1.1	95 ± 3.3	212.4 ± 2.7	70 ± 2.3
3	1.34 ± 1.2	94.1 ± 3.3	254.4 ± 4.8	61 ± 4.9
4	1.44 ± 1.3	93 ± 3.6	155.5 ± 3.5	82 ± 5.3
1-Deoxynojrimycin	–	–	–	–
Orlistat	28.9 ± 6.4	92 ± 6.4	–	–
Acarbose	–	–	230.85 ± 4.1	68 ± 4.7

Note: Results are given as mean values $\pm$ SD of $n = 3$ independent experiments.

50 µg/kg (Kumar et al., 2013). These results suggest the enzyme inhibitory activity of these compounds which were isolated from NPF are likely contributors to anti-hyperglycemic and anti-hyperlipidemic effects observed as a result of NPF treatment in the in vivo study. To further support our findings, Wang et al. (2017).reported that stigmasterol when orally administrated to the KK-Ay mice significantly improved insulin resistance and oral glucose tolerance while reducing fasting blood-glucose levels and blood lipid parameters: triglyceride and cholesterol Moreover, Ramu et al. investigated the antihyperglycemic of stigmasterol and reported that it caused a decrease in fasting glucose levels in addition to improvement of diabetic symptoms such as polyuria, polydipsia and urine glucose (Ramu et al., 2016). In a different study, friedelin showed a significant lipid-lowering effect restoring plasma levels of total cholesterol, triacylglycerides,

HDL, and LDL to their normal levels (Duraipandiyan et al., 2016). It is noteworthy that this is the first study reporting pancreatic lipase and α-amylase inhibition by calotroproceryl acetate A (**1**) and calotroprocerol A (**3**) which were isolated from *P. vulgaris* pods. Together, these four compounds can be responsible, at least partially, for the antidiabetic effect observed for NPF.

4 | CONCLUSION

Our study indicates that both MSC and NPF of *P. vulgaris* pods have a potent antidiabetic activity on their own and a remarkable synergistic effect when used in combination. LC/MS analysis of NPF of *P. vulgaris* indicated its enrichment with antioxidant phenolic compounds including flavonoids (quercetin, acacetin, apigenin, luteolin and diadzein) and phenolic acids such as sinapic, caffeic and coumaric acids. In addition, four bioactive triterpenoids were identified in NPF and found to be good inhibitors of both pancreatic lipase and α-amylase. Therefore, it can be argued that NPF of *P. vulgaris* extract can potentiate MSC antidiabetic activity directly by exerting its own antidiabetic effect and indirectly by increasing the homing of the MSC in the pancreas.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

Shahira Ezzat: Conceptualization; Data curation; Supervision; Visualization; Writing – original draft; Writing – review & editing. **Mohamed F. Abdel Rahman:** Data curation; Methodology; Resources; Supervision; Writing – original draft. **Maha M. Salama:** Data curation; Formal analysis; Investigation; Visualization; Writing – original draft. **Engy A. Mahrous:** Conceptualization; Data curation; Resources; Writing – review & editing. **Amany El Bariary:** Data curation; Investigation; Writing – original draft.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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