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Exploring pulegone in type 2 diabetes treatment: Molecular docking, influence on insulin resistance and modulation of pro/anti-inflammatory cytokines

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Abstract: **Background**: Type 2 diabetes involves insulin resistance, where muscle, liver, and fat cells fail to utilize glucose leading to hyperglycemia. **Objectives**: This study explores the therapeutic potential of pulegone in type 2 diabetes by examining its molecular interactions with key metabolic targets and evaluating its effects on insulin resistance and inflammatory cytokine modulation. **Methods**: Except for normal controls, animals were fed a high-sucrose, high-fat diet for four months to induce insulin resistance. Treatment groups received metformin (150 mg/kg) or pulegone doses: low (5 mg/kg), medium (10 mg/kg), and high (15 mg/kg) for 45 days. Post-treatment, tissues and blood were collected. Blood serum was analyzed for liver markers, lipid profile, and blood for glycated hemoglobin. ELISA was used for cytokines (resistin, adiponectin, IL-1Ra, TNF-α), and qPCR assessed gene expression of NF-κB, TNF-α, PPAR-α/γ, IL-6, IL-10, IL-1Ra, and adiponectin. Histopathology was performed. Data were analyzed using GraphPad Prism. Molecular docking was conducted to identify the binding affinities of pulegone to target proteins. **Results**: Pulegone significantly reduced fasting blood glucose, glycated hemoglobin, triglycerides, cholesterol, and liver enzymes, while increasing HDL levels compared with diabetic controls. It also decreased serum resistin, TNF-α, IL-6 and NF-κB expression, while upregulating adiponectin, IL-10, PPAR-α, PPAR-γ and IL-1Ra. Histopathological examination showed protective effects on the pancreas, liver, kidney, heart, and aorta. Docking analysis favored strong binding affinities of pulegone with key targets. **Conclusion**: Pulegone ameliorates insulin resistance by improving glycemic and lipid profiles, modulating pro- and anti-inflammatory cytokines, and protecting vital organs, suggesting its potential as a multitarget antidiabetic agent.

Keywords: Anti-inflammatory cytokines; Metformin; Pulegone; Pro-inflammatory; Type 2 diabetes

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic illness that is widespread and complex, defined by high plasma glucose levels (Tattersall and Matthews, 2024). DM comprises two main types: Type 1 diabetes (T1D) and Type 2 diabetes (T2D). T1D is typically diagnosed in childhood or adolescence, arises from an autoimmune response that progressively destroys pancreatic beta cells responsible for insulin production (Chandrasekaran and Weiskirchen, 2024). In contrast, T2D, often associated with lifestyle factors such as obesity, involves a combination of endogenous resistance to insulin action and potential declines in pancreatic beta-cell function, resulting in chronic hyperglycemia and increased risk of complications (Ruze *et al.*, 2023).

Effective management of diabetes requires tailored strategies, including lifestyle modifications, pharmacotherapy and, in some cases, insulin therapy, underscoring the importance of understanding the unique pathogenesis of each type to optimize treatment and prevent complications (Antar *et al.*, 2023). Insulin is a critical regulator of growth and glucose metabolism. It works by attaching to the insulin receptor on the plasma membrane, which starts a complex signaling cascade (Sasako, 2024). The phosphorylation of insulin receptor substrates, mainly IRS-1, on tyrosine residues results from this interaction's enhancement of insulin receptor tyrosine kinase activity. The phosphorylation of IRS proteins initiates two main signaling pathways: the Ras-mitogen-activated protein kinase (MAPK) pathway, which mainly controls insulin's effect on growth, mitogenesis and differentiation and the phosphatidylinositol-3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway, which is principally in charge of mediating insulin's actions on nutrient metabolism. Many insulin-resistant diseases are linked to abnormalities in the insulin-signaling system. The activation of pathways and factors (e.g., protein kinase C, c-jun N-terminal kinase [JNK], reactive oxygen species,

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nuclear factor κB [NFκB] pathway, protein phosphatase A2 [PPA2] and cytokines that antagonize insulin signaling by diminishing the activating phosphorylation of insulin-signaling proteins is one of the mechanisms that have been proposed to link lipid metabolites to reductions in insulin signaling (Khalilov and Abdullayeva, 2023). Various classes of antidiabetic medications are currently employed in the management of diabetes, each having adverse effects. Common issues include low blood sugar (hypoglycemia), weight gain and stomach problems. Certain newer drugs, like SGLT2 inhibitors, might increase the risk of infections or dehydration, while GLP-1 agonists could cause nausea or, in rare cases, pancreatitis (Khan *et al.*, 2023).

Phytotherapy, which refers to the use of plant-based medicines, has a rich history as a source of medicinal remedies and it has been explored extensively over the years for its potential in treating diabetes (Onukwuli *et al.*, 2024).

Pulegone, a compound with promising therapeutic properties, exhibits analgesic effects and has been traditionally used to alleviate pain and discomfort, including headaches and muscle aches. Its anti-inflammatory properties make it a candidate for managing inflammatory conditions such as arthritis and inflammatory bowel disease (IBD), with potential to reduce associated symptoms (Hudz *et al.*, 2023). Pulegone's muscle relaxant and antispasmodic effects suggest its application in managing conditions characterized by muscle spasms and cramps (Ribeiro-Silva *et al.*, 2022). *Mentha spicata* leaf aqueous extract, which is high in pulegone, showed notable hypoglycemic and antioxidant properties in a study involving diabetic rats. The extract improved lipid profiles and decreased blood glucose levels, indicating that it may be used to treat oxidative stress and hyperglycemia associated with diabetes. Pulegone, a major monoterpenoid constituent of *Mentha pulegium* essential oil (MPEO), has demonstrated promising antidiabetic activity through both in vitro and in silico evaluations (El Omari *et al.*, 2025). This study was performed to evaluate the *in-vivo* effect of pulegone in insulin-resistant rats by measuring various cytokines involved in type 2 diabetes pathophysiology using a high-fat, high-sucrose diet.

MATERIALS AND METHODS

Chemicals and reagents

Phytocompound pulegone was bought from Sigma-Aldrich (Cat# 04620590, St. Louis, MO, USA). ELISA assay kits for Resistin (Cat# EH0269), Adiponectin (Cat# EH2593), IL-1Ra (Cat# EH0172) and TNF-α (Cat# EH0302) were obtained from FineTest Biotech, Wuhan, China. Ketamine was acquired from Sigma-Aldrich (Cat# K1884, Sigma-Aldrich, St. Louis, MO, USA), while a cDNA synthesis kit was sourced from ABclonal (Cat# RK20400, Woburn, United States). RIPA lysis buffer for RNA extraction was purchased from Elabscience (Cat# E-BC-R327, Wuhan, China). Other chemicals, including cholesterol (Cat# C8667), cholic acid (Cat# 135240) and H&E staining reagents (Cat# H3136), were also obtained from Sigma-Aldrich. Metformin (Cat# 317240), used as a standard drug, was similarly purchased from Sigma-Aldrich, St. Louis, MO, USA.

Animals

Wistar rats (180-200 g) were procured from the animal house and housed at the University of Lahore, Pakistan, after obtaining approval from the University Research Ethics Committee (IREC-2022-26), which is compliant with the ARRIVE 2.0 Guidelines and national and international norms. The rats were maintained in clean, spacious polyacrylic cages with a 12-hour day/night cycle, 70% humidity and received a standard normal pellet diet (NPD) and water.

Composition of high sucrose high fat diet (HSFD)

Wistar rats were fed from trays (100g) kept in their cages. The rats were divided into two groups: the normal control (NC) group, which received a normal pellet diet and water throughout the study and the HSFD group, which was fed a high-sucrose and high-fat diet for four months (Frick *et al.*, 2023). Each 100 g of HSFD consisted of 50 g of normal diet, 40 g sucrose, 4 g cholesterol, 2 g cholic acid and 4 g egg yolk powder (Wang *et al.*, 2020).

Induction

Induction of insulin resistance was achieved by maintaining the animals on a high-sucrose and high-fat diet for four months (Lei *et al.*, 2023). Fasting plasma glucose (FPG) levels were measured and values ≥ 300 mg/dl were considered indicative of diabetes. Hemoglobin A1c (HbA1c) levels were also assessed to monitor average blood glucose levels over the past 3 to 4 months, aiding in the diagnosis and prediction of prediabetes and insulin resistance (Weiner *et al.*, 2023).

Experimental design

The doses of pulegone selected for this study (5, 10 and 15 mg/kg/day) were chosen based on safety, with the potential to detect pharmacological effects. Previous toxicological investigations in rats have shown that repeated administration of pulegone at ≥ 20-40 mg/kg/day is associated with hepatotoxicity and renal alterations, while lower exposures generally remain within the no-observed-adverse-effect level (NOAEL) range (Thorup *et al.*, 1983). Based on this evidence, we deliberately selected doses below 20 mg/kg/day to ensure animal safety while still exploring a biologically meaningful range.

Wistar rats were divided into six groups (n = 6 each) (Lin *et al.*, 2024):

• Group 1 Normal control rats (NC) received normal saline (1 mL/kg) orally.
• Group 2 HSFD rats (diabetic control) fed a high-sucrose and high-fat diet for four months.

• Group 3 HSFD (positive control) diabetic rats received metformin 150 mg/kg.
• Group 4 HSFD diabetic rats received pulegone 5 mg/kg.
• Group 5 HSFD diabetic rats received pulegone 10 mg/kg.
• Group 6 HSFD diabetic rats received pulegone 15 mg/kg.

All groups utilized normal saline as the vehicle. After the 165-day experimental period, rats were anesthetized and Samples of tissue and blood were obtained for additional analysis (Oliveira *et al.*, 2020). Overnight, rats were denied food, but they were allowed unlimited access to water. After being intraperitoneally anesthetized with Ketamine (91 mg/kg) and xylazine (9.1mg/kg), further analysis was carried out (Navarro *et al.*, 2021). 5 ml of blood was obtained by heart puncture. Serum was collected for the determination of different parameters. Internal organs (liver, kidney, pancreas, heart and aorta) were also collected for histopathological analysis.

Estimation of body weight
Body weight of HSFD-fed rats was monitored on Day 0, Day 28, Day 56, Day 84, Day 112, Day 140 and Day 165 during the experimental period for all groups.

Fasting blood glucose (FBG)
FBG was evaluated from blood samples collected from the tail of the animal with a glucometer AlphaTRAK® (Abbot Laboratory) (El *et al.*, 2023) on Day 0, Day 28, Day 56, Day 84, Day 112, Day 140 and Day 165 in NC, DC and treated groups (pulegone 5 mg/kg, pulegone 10 mg/kg, pulegone 15 mg/kg and metformin 150 mg/kg).

Biochemical analysis
Blood samples were taken through intracardiac puncture and transferred to the UOL Diagnostic Lab for biochemical analysis. Serum was collected for further analysis of lipid and liver biochemical parameters. Total cholesterol, triglycerides, LDL and HDL of NC and HSFD rats were determined spectrophotometrically using an automated biochemical analyzer. Liver Function Tests (LFTs), such as ALT and AST levels of NC and HSFD rats, were determined spectrophotometrically using a Roche Cobas C111 Chemistry Analyzer. HbA1c level was measured from blood using a Roche Cobas C111 Chemistry Analyzer.

Determination of serum levels of resistin, adiponectin, IL-1Ra and TNF-α through ELISA
The levels of Resistin, Adiponectin, IL-1Ra and TNF-α were measured using ELISA kits (Batch no.R1091E117 J and R1309E117 J).

Quantitative polymerase chain reaction (RT-PCR)
RTPCR was conducted for rat Resistin, Adiponectin, TNF-α, IL-1Ra, IL-6, IL-10, PPARα, PPARγ and NF-κB using specific primers. RNA extraction using TRIzol reagent was carried out in accordance with the manufacturer's instructions. A Nanodrop 1000 spectrophotometer was used to quantify the extracted mRNA. A reverse transcriptase kit from Thermo Fisher Scientific, USA, was used to produce cDNA. A Maxima SYBR green for qPCR kit from Thermo Fisher Scientific, USA was used. The reference gene utilized was GAPDH. The 2-ΔΔCT technique was employed to ascertain the relative expression. Based on previously released information, the primers were designed (Hazarika *et al.*, 2023).

The sequences of the primers and their annealing temperatures are shown in table 1.

Histopathology
Histological assessments of the pancreas, liver, kidney, heart and aorta were conducted to detect any structural changes. Tissue sections were stained with eosin followed by hematoxylin for microscopic examination at 10X and 40X magnification.

Molecular docking analysis
Ligand preparation
The canonical SMILES for the ligands, such as pulegone and metformin, were retrieved from the PubChem public database. The molecular builder feature in MOE was used to place the canonical SMILES of ligands into the MOE workspace. The first step was to ensure the ligands had stable three-dimensional shapes and give them partial charges with the AMBER (Assisted Model Building and Energy Refinement) force field to improve interaction prediction during docking (Mohamed *et al.*, 2022).

Receptor preparation
The three-dimensional structures of the targeting proteins, including tumor necrosis factor-alpha (PDB ID: 2AZ5), peroxisome proliferator-activated receptor-alpha (PDB ID: 2ZNN), peroxisome proliferator-activated receptor-gamma (PDB ID: 1I7I) and interleukin-1 receptor antagonist (PDB ID: 1ILT), resistin (PDB ID: 1RGX), adiponectin (PDB ID: 4DOU), interleukin-6 (PDB: 1ALU), interleukin-10 (PDB ID: 2ILK) and nuclear factor kappa-B (NF-kB PDB ID: 1IKN) were downloaded from the Protein Data Bank (PDB) database. The process for preparing receptors in MOE involved loading PDB files, deleting any unnecessary chains, removing water molecules and other ligands and producing clean receptors. After that, hydrogen atoms were added to the structure to enhance docking simulations and essential residues were assigned the protonation state that corresponds to the normal pH of the body (Ali *et al.*, 2024).

Docking setup and analysis
Docking was set up using several essential factors. The optimal binding pockets on the receptors were found using MOE's built-in Site Finder tool and a grid box was created to include these areas. The Triangle Matcher was chosen as the docking method due to its effectiveness in producing

initial poses and the Induced Fit Technique was used to further refine the docking results. The MOE interface was used to start the docking simulation, applying several crucial parameters that ensured an accurate representation of ligand-receptor interactions. An extensive sampling of potential ligand orientations was ensured by conducting 100 docking attempts in total. The best docked ligand conformations within the receptor binding sites were identified by evaluating the binding affinities of the produced poses using MOE's default scoring mechanism (Liu *et al.*, 2023). The outcomes of the simulations were evaluated by post-docking assessments. The visualization tools of MOE and the Discovery Studio Visualizer 2021 Client 21.1.0 were used to visualize the docked ligand conformations inside the binding sites for better representation (Baroroh *et al.*, 2023). The most advantageous positions were determined by comparing the binding scores as part of the binding affinity evaluation. In order to get insight into the binding mechanisms between the ligands and their receptors, an interaction analysis was also conducted to look at important interactions, such as hydrogen bonds and hydrophobic contacts.

Statistical analysis

For each group of six rats, the data are shown as the mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used to establish statistical significance and Bonferroni's post-hoc test was then performed. All analyses were conducted using GraphPad Prism software (version 8), with significance set at $p < 0.05$.

RESULTS

Effect on body weights

Significant decreases were observed in the DC group compared to the NC. Whereas, treatment groups and metformin showed not as much of weight reduction as compared to diseased group (Fig. 1a).

Effect on blood glucose levels

Pulegone treatments effectively improved insulin resistance and glycemic control in HSFD-fed rats, significantly lowering blood glucose levels compared to DC rats. NC rats maintained normal glucose levels throughout the study (Fig. 1b).

Biochemical analysis

Effect on lipid profile

Diabetic control rats showed elevated triglyceride (TG) levels, whereas treatment with pulegone and metformin mitigated these increases (Table 2).

Effect on liver function tests (LFTs)

Treatment with pulegone and metformin reduced serum alanine transferase (ALT) and aspartate transaminase (AST) levels as compared to DC rats (Table 2).

Effect on HbA1c%

Pulegone treatments lowered HbA1c% levels compared to DC, demonstrating improved long-term glycemic control (Table 2).

Determination of serum levels of resistin, adiponectin, IL-1Ra and TNF-α

The level of adiponectin significantly increased while resistin, IL-1Ra and TNF-α were significantly decreased at different doses, pulegone 5 mg/kg, pulegone 10 mg/kg and pulegone 15 mg/kg (Fig. 2).

Quantification of mRNA levels of resistin, adiponectin, TNF-α, IL-1Ra, IL-6, IL-10, PPARα, PPARγ and NF-κB

The mRNA expression levels of cytokines resistin, TNF-α, IL-1Ra and IL-6 were higher in disease control on the other hand mRNA expression levels decreased in these cytokines but adiponectin, IL-10, PPARα, PPARγ and NF-κB expression levels increased as compared to disease control at different doses of pulegone (pulegone 5 mg/kg, pulegone 10 mg/kg, pulegone 15 mg/kg). (Fig. 3)

Histopathological studies

Histological examination revealed normal tissue structures in NC rats, whereas HSFD-fed rats exhibited significant pathological changes, including pancreatic islet cell loss, hepatocyte damage and kidney tubule abnormalities. Treatment with pulegone and metformin ameliorated these pathological changes, indicating protective effects on pancreatic, heart, kidney, liver and aortic tissues. Histopathological examination of pancreatic, heart, kidney, liver and aortic tissues (Fig. 4).

Histopathological analysis

(1A) pancreatic tissues normal control shows no pathological changes, (1B) in disease control less number of Beta cells and information was observed, (1C) in met treaded, (1D)P1, (1E)P2 and (1F)P3 near to normal Beta cells concentration and no evidence of any degeneration. (2A) normal cardiac tissue (2B) fibrosis was seen (2C) cardiac tissue of standard drug treatment revealed normal myofibers (2D)P1,(2E)P2and (2F)P3 cardiac tissue of pulegone treatment also revealed near normal myofibers. (3A) Normal nephrons and glomerulus are healthy (3B) diabetic kidney showing shrinkage of tubule (3C) kidney of standard drug treatment revealed normal nephrons (3D) P1, (3E) P2 and (3F) P3 no tubular atrophy seen, tubular brush borders seen normal, glomerulus is healthy, no vascularization of renal cells. (4A) Non-diabetic section of liver showing normal hepatocytes (4B) Liver of diabetic rat showing loss of the normal architecture with the distended portal vein, fibrosis, leucocytic inflammation (4C) showing near normal hepatocytes (4D) P1 (4E)P2 (4F)P3 Diabetic rats treated with pulegone show near normal hepatocytes, mild sinusoidal dilatation around central vein when compared to liver of rat not treated. (5A) aortic tissues are normal (5B), tunica intima and tunica media are not of

normal thickness (5C), aortic tissue of standard drug treatment revealed normal tunica intima (5D). P1 (5E) P2 (5F)P3 tunica intima and tunica media are of normal thickness. (P1)5mg/kg, (P2)10mg/kg, (P3)15mg/kg of pulegone. All the pictures' magnification is ×400. (40 × 10).

Pulegone docking affinities and scores

The molecular docking analysis was performed to evaluate the binding affinities of pulegone and metformin across various receptor targets. For TNF-alpha (PDB ID: 2AZ5), pulegone demonstrated a binding score of -4.14 kcal/mol, with a hydrogen-acceptor interaction at LYS 11. The RMSD value of 1.47 Å indicates a stable interaction. Metformin exhibited a stronger binding score of -4.50 kcal/mol, interacting with ASN 34 and LEU 93 via multiple hydrogen bonds, with bond distances ranging from 2.87 Å to 3.23 Å and a lower RMSD of 0.87 Å, suggesting a more accurate docking pose. In the case of PPAR-α (PDB ID: 2ZNN), pulegone displayed a favorable docking score of -5.50 kcal/mol, binding to MET 355 and LYS 358 with distances of 3.72 Å and 3.99 Å, respectively. Its RMSD of 0.91 Å suggests a high-confidence docking. Metformin showed no significant interactions with PPAR-α, suggesting limited affinity for this receptor. For PPAR-γ (PDB ID: 1I7I), pulegone exhibited a robust binding score of -5.62 kcal/mol, with a hydrogen-acceptor interaction at TYR 473 and a low RMSD of 0.98 Å, indicating a stable and accurate docking pose. Metformin, with a binding score of -4.97 kcal/mol, interacted with HIS 323 at 3.03 Å, though its higher RMSD of 1.69 Å implies a less accurate binding compared to pulegone. In the IL-1Ra (PDB ID: 1ILR) receptor, pulegone showed a binding score of -4.76 kcal/mol with an interaction at LEU 30, which suggests a strong affinity for this receptor. The RMSD value of 1.25 Å is favorable for reasonable docking pose. Metformin had a weaker binding affinity with CYS 69 at 1.43 Å and a docking score of -4.56 kcal/mol, suggesting fewer binding affinities. For resistin (PDB ID: 1RGX), pulegone bound effectively to LEU 90 with a hydrogen-acceptor interaction at 2.94 Å, yielding a docking score of -5.00 kcal/mol and an RMSD of 1.08 Å. Metformin exhibited a less favorable binding score of -4.66 kcal/mol, with hydrogen bonds at distances of 2.98 Å and 3.19 Å, respectively, suggesting a weaker interaction compared to pulegone. In the adiponectin (PDB ID: 4DOU) receptor, pulegone showed a binding score of -4.353 kcal/mol, interacting with GLN 280 via a hydrogen-acceptor bond at 3.03 Å, with an RMSD of 0.83 Å. Metformin demonstrated weaker interactions with ASP 242 and TYR 278, indicating less favorable docking compared to pulegone . For the IL-6 (PDB ID: 1ALU) receptor, pulegone exhibited a binding score of -4.18 kcal/mol, with an H-acceptor interaction at LEU 64 and an RMSD of 1.33 Å. Metformin had comparable binding score of -4.18 kcal/mol, interacting with ASP 63. In the IL-10 (PDB ID: 2ILK) receptor, pulegone bound to PHE 146 through an H-pi interaction at 3.77 Å, with a docking score of -4.48 kcal/mol and an RMSD of 1.52 Å. Metformin exhibited a stronger binding score of -4.81 kcal/mol, with hydrogen bonds to GLU 96 at distances of 2.84 Å and 2.77 Å, suggesting slightly better interaction with IL-10 than pulegone. For the NF-kB (PDB ID: 1IKN) receptor, pulegone displayed a docking score of -5.34 kcal/mol, binding to GLN 29 and LYS 221 with distances of 3.28 Å and 2.87 Å, respectively. Metformin showed a slightly lower binding score of -5.05 kcal/mol, interacting with ALA 242 at 3.11 Å (Table 3) (Fig. 5). Pulegone exhibits promising binding affinities across several key receptors, demonstrating its potential for modulating inflammatory and metabolic pathways. Metformin also shows significant docking interactions, particularly with TNF-alpha, IL-10 and NF-kB. Pulegone appears to have a stronger affinity for many receptors, indicating its potential as a therapeutic candidate for inflammation and metabolic regulation.

DISCUSSION

This study demonstrates that pulegone effectively reverses insulin resistance in insulin-resistant rats fed a high-sucrose high-fat diet. Insulin resistance was induced by this diet, leading to notable changes in body weight, blood glucose levels, lipid profiles, liver function tests (LFTs) and HbA1c compared to control groups. Rats on the high-sucrose high-fat diet exhibited increased body weights compared to non-diabetic controls, likely due to increased body fat accumulation (Ramos-García *et al.*, 2021). These rats also showed elevated blood glucose levels after four months, indicative of pancreatic β-cell damage. Treatment with pulegone significantly lowered glucose levels, demonstrating good glucose control compared to standard metformin treatment.

The diabetic control group exhibited elevated triglyceride (TG) levels, which are implicated in cardiovascular disease (Akivis *et al.*, 2024 ; Haring *et al.*, 2024). Treatment with pulegone effectively maintained lipid profiles, with significant reductions in serum cholesterol levels observed in pulegone-treated groups compared to diabetic controls. Both pulegone and metformin treatments showed promise in managing dyslipidemia associated with diabetes, essential for preventing cardiovascular complications. Assessment of liver enzymes (ALT and AST) indicated increased levels in untreated diabetic rats, suggesting liver cell damage (Han *et al.*, 2024). Pulegone treatment demonstrated hepato-protective effects comparable to metformin, reducing enzyme levels possibly through antioxidant and radical scavenging properties(Juncos *et al.*, 2024).

Evaluation of HbA1c levels revealed elevated levels in the diabetic control group, indicative of poor glycemic control and elevated blood glucose.

Table 1: Shows sequences of the primers and their annealing temperatures.

Gene	Primers	Length bp	Tm	product size
IL10	F: GCCCAGAAATCAAGGAGCATT	20	60	181bp
	R: CAGCTGTATCCAGAGGGTCTTC	20	62	
PPAR-α	F: ACGATGCTGTCCTCCTTGATG	20	60.5	217bp
	R: GCGTCTGACTCGGTCTTCTTG	20	60.5	
PPAR-ɣ	F: CCCTTTACCACGGTTGATTTCTC	24	62	237bp
	R: CAGGCTCTACTTTGATCGCACT	24	60	
Adipoq	F: CCACCCAAGGAAACTTGTGC	20	60.5	136bp
	R: GACCAAGAACACCTGCGTCT	20	60.5	
IL1ra	F: GACCTGTGCCAGTCTATGGG	20	62.5	135bp
	R: GATGCCCTGGTGGGTTTCAT	20	60.5	
TNF-α	F: ATGGGCTCCCTCTCATCAGT	20	60.5	106bp
	R: GCTTGGTGGTTTGCTACGAC	20	60.5	
NFkB1	F: CTGAGTCCCGCCCCTTCTAA	20	60	217bp
	R: CCTCTGTGTAGCCCATCTGTC	20	59	
IL6	F: CCTACCCCAACTTCCAATGCTC	20	60	270bp
	R: GGATGGTCTTGGTCCTTAGCC	20	60	
GAPDH	F: GCCCAGCAAGGATACTGAGA	20	60.5	164bp
	R:TATTGATGGTATTCGAGAGAAGGG	24	62	

Table 2: Effects of pulegone on blood biochemical parameters in insulin-resistant rats.

Variables	Control	Disease	Met treated	HSFD+P5mg/kg	HSFD+P10mg/kg	HSFD+P15mg/kg
			Mean ± S.E.M			
AST U/I	61.00±4.382	167.5±18.09###	85.67±5.886***	76.67±5.976***	82.00±5.046***	97.67±8.480***
ALT U/I	26.50±3.019	81.00±6.73###	37.67±2.201***	39.33±2.860***	40.50±1.708***	44.17±2.822***
HbA1C%	4.55±0.33	12.48±0.38###	4.367±0.156***	8.083±0.338***	6.300±0.438***	3.767±0.260***
Cholesterol mg/dl	45.96±1.54	160.2±21.68###	53.40±2.108***	47.02±1.996***	45.14±1.591***	44.16±0.676***
Triglycerides mg/dl	59.93±1.83	110.8±10.43###	64.15±1.51***	43.82±1.92***	43.51±1.61***	44.86±1.99***
HDL mg/dl	38.09±2.376	15.65±1.804###	35.57±1.567***	36.20±2.916**	37.48±3.176*	35.51±3.103*
LDL mg/dl	51.06±3.568	138.5±37.15##	51.77±2.349**	54.12±1.941**	39.37±10.72***	57.80±2.411**
VLDL mg/dl	23.06±1.185	160.0±27.38###	50.08±7.794***	61.17±7.867***	30.50±3.713***	24.67±1.229***

Table 2: Statistical data was applied by one way analysis of variance (ANOVA) and post hoc Tukey's test for multiple comparison test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in contrast to the diabetic control group shows significance, where ***reflects the highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control. All biochemical parameters show significant improvement as compared to the disease group.

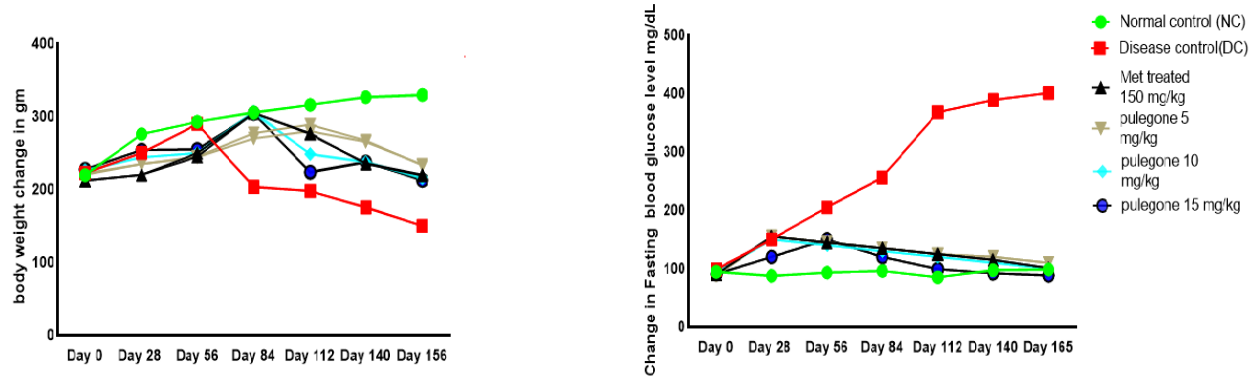

Fig. 1: (a) Body weight (b) Fasting blood glucose levels changes in all groups, including NC, DC and treated groups (pulegone 5 mg/kg, pulegone 10 mg/kg, pulegone 15 mg/kg, and metformin 150 mg/kg). Data is presented as the mean ± SD ($n = 6$). Statistical analysis was applied by using one way analysis of variance (ANOVA) and post hoc multiple comparison test. The body weight of DC gradually decreased, while others tended towards normal. The glucose levels significantly increased in DC, whereas they remained normal in NC, but tended to decrease in the treated ones as compared to DC.

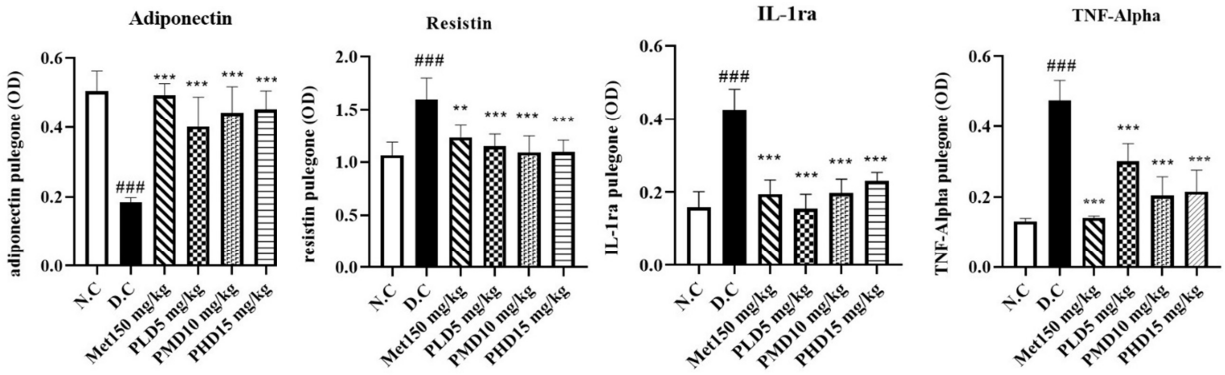

Fig. 2: Statistical data was applied by one way analysis of variance (ANOVA) and post hoc Tukey's test for multiple comparison test. Cytokines values are presented as mean ± SEM for n = 6. * $p < 0.0001$ relative to the control group with diabetes. + $p < 0.0001$ relative to the control group, where *** denotes the greatest degree of significance and *, ** and *** represent significance levels of $p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively. The difference between the two control groups, disease control and normal control, is depicted in ####. Pulegone showed significant effects in reducing IR.

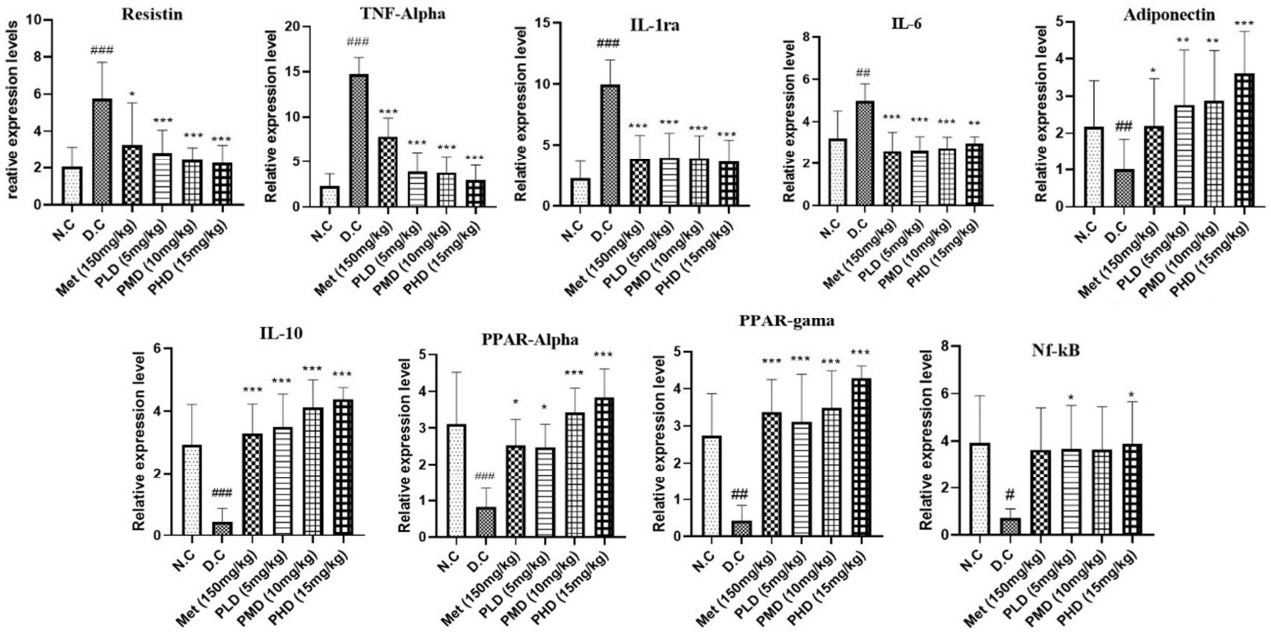

Fig. 3: Statistical data was applied by one way analysis of variance (ANOVA) and post hoc Tukey's test for multiple comparison test. PPAR-α(A), IL-10 (B), IL-6 (C), IL-1ra(D), PPAR-γ (E), Nf-Kb(F), Resistin(G), TNF-α(H) and Adiponectin(I) values are shown as the mean ± SEM (n = 6). * $p < 0.0001$ in comparison to the diabetic control group: * = $p < 0.05$ ** = $p < 0.001$, *** = $p < 0.0001$ shows significance, where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control. Pulegone successfully decreased IR.

Treatment with Pulegone and metformin led to reductions in HbA1c levels comparable to standard treatment, highlighting their effectiveness in improving long-term glycemic control.

At least in rat models, resistin has been linked to the pathophysiology of obesity-mediated insulin resistance and Type II diabetes mellitus. When combined, resistin and Adiponectin have opposing effects, which implies that their combination may influence an obese person's metabolic profile and lead to issues associated with obesity. Several other adipocytokines may have different roles in raising the risk of disease (Abudalo *et al.*, 2024). Furthermore, resistin also promotes inflammation (Bârsan *et al.*, 2024). However, there has been a great deal of debate up to this point regarding the physiological significance of this 12.5 kDa polypeptide in both rat and human systems.

Table 3: *In-silico* molecular docking analysis of pulegone in the binding pockets of different key targets

Compound	S score (kcal/mol)	RMSD (Å)	Atom of Compounds	Atom of receptors	Residue of receptor	Type of interaction bond	Distance (Å)
			TNF-alpha (PDB ID: 2AZ5)				
Pulegone	-4.14	1.47	O-1	N	LYS 11 (C)	H-acceptor	3.08
Metformin	-4.50	0.87	N-15	O	ASN 34 (B)	H-donor	2.87
			N-18	O	LEU 93 (A)	H-donor	3.05
			N-18	O	ASN 34 (B)	H-donor	3.23
			PPAR-α (PDB ID: 2ZNN)				
Pulegone	-5.50	0.91	C-25	SD	MET 355 (A)	H-donor	3.72
			O-1	CE	LYS 358 (A)	H-acceptor	3.99
Metformin	-4.86	1.22			No interaction		
			PPAR-γ (PDB ID: 1I7I)				
Pulegone	-5.62	0.98	O-1	OH	TYR 473 (A)	H-acceptor	3.09
Metformin	-4.97	1.69	N-15	NE2	HIS 323 (A)	H-donor	3.03
			IL-1Ra (PDB ID: 1ILR)				
Pulegone	-4.76	1.25	O-1	N	LEU 30 (B)	H-acceptor	3.30
Metformin	-4.56	1.43	N-18	O	CYS 69 (A)	H-donor	3.28
			Resistin (PDB ID: 1RGX)				
Pulegone	-5.00	1.08	O-1	N	LEU 90 (A)	H-acceptor	2.94
Metformin	-4.66	0.63	N-18	O	LYS 29 (C)	H-donor	2.98
			N-11	N	LEU 90 (A)	H-acceptor	3.19
			Adiponectin (PDB ID: 4DOU)				
Pulegone	-4.33	0.83	O-1	NE2	GLN 280 (A)	H-acceptor	3.22
Metformin	-4.50	1.32	N-15	OD1	ASP 242 (A)	H-donor	2.65
			N-13	OH	TYR 278 (A)	H-acceptor	3.16
			IL-6 (PDB ID: 1ALU)				
Pulegone	-4.18	1.33	O-1	N	LEU 64 (A)	H-acceptor	3.00
Metformin	-4.18	1.27	N-18	O	ASN 63 (A)	H-donor	3.12
			IL-10 (PDB ID: 2ILK)				
Pulegone	-4.48	1.52	C-5	6-ring	PHE 146 (A)	H-pi	3.77
Metformin	-4.81	1.28	N-15	OE2	GLU 96 (A)	H-donor	2.84
			N-18	OE2	GLU 96 (A)	H-donor	2.77
			NF-KB (PDB ID: 1IKN)				
Pulegone	-5.34	1.71	C-25	OE1	GLN 29 (A)	H-donor	3.28
			O-1	NZ	LYS 221 (A)	H-acceptor	2.87
Metformin	-5.05	1.30	N-15	O	ALA 242 (A)	H-donor	3.11

The levels of resistin were increased in diabetic rats and its levels were significantly decreased in the treated rat groups.

The expression of adiponectin is exclusive to adipose tissue and declines as obesity increases (Andarianto *et al.*, 2024). Insulin resistance lowers the levels of adiponectin since it aids in insulin sensitivity(Lu *et al.*, 2024). Adiponectin levels in circulation were lower in subjects who were obese in the abdomen ($P < 0.001$). The BMI was inversely correlated with the level of circulating adiponectin(Ma *et al.*, 2024). In this study, adiponectin levels decreased in insulin-resistant rats, while the levels of adiponectin increased in pulegone-treated groups. Furthermore, adiponectin is dysregulated as a result of hyperglycemia.

While growth factor loses its ability to control adiponectin production, it mostly affects adiponectin levels; growth factor 21 has a positive response to PPAR gamma and is further elevated by hyperglycemia. It is well established that PPAR gamma ligands contribute to weight loss.

Adipocytokine tumour necrosis factor alpha (TNF-α) induces the acute phase response and contributes to systemic inflammation(Park and Han, 2024). The main cells that release TNF-α are macrophages, but a wide range of other cells, including adipocytes, also do so. TNF-α affects glucose metabolism and inhibits insulin transduction (Charaslertrangsi *et al.*, 2025). Type 2 diabetes mellitus may be influenced by disruptions in TNF-α metabolism, as these disturbances have been linked to metabolic diseases such as obesity and insulin resistance (Alzamil, 2020). In our work, TNF-α levels were elevated in insulin-resistant rats, but their expression levels declined when pulegone treatment was given.

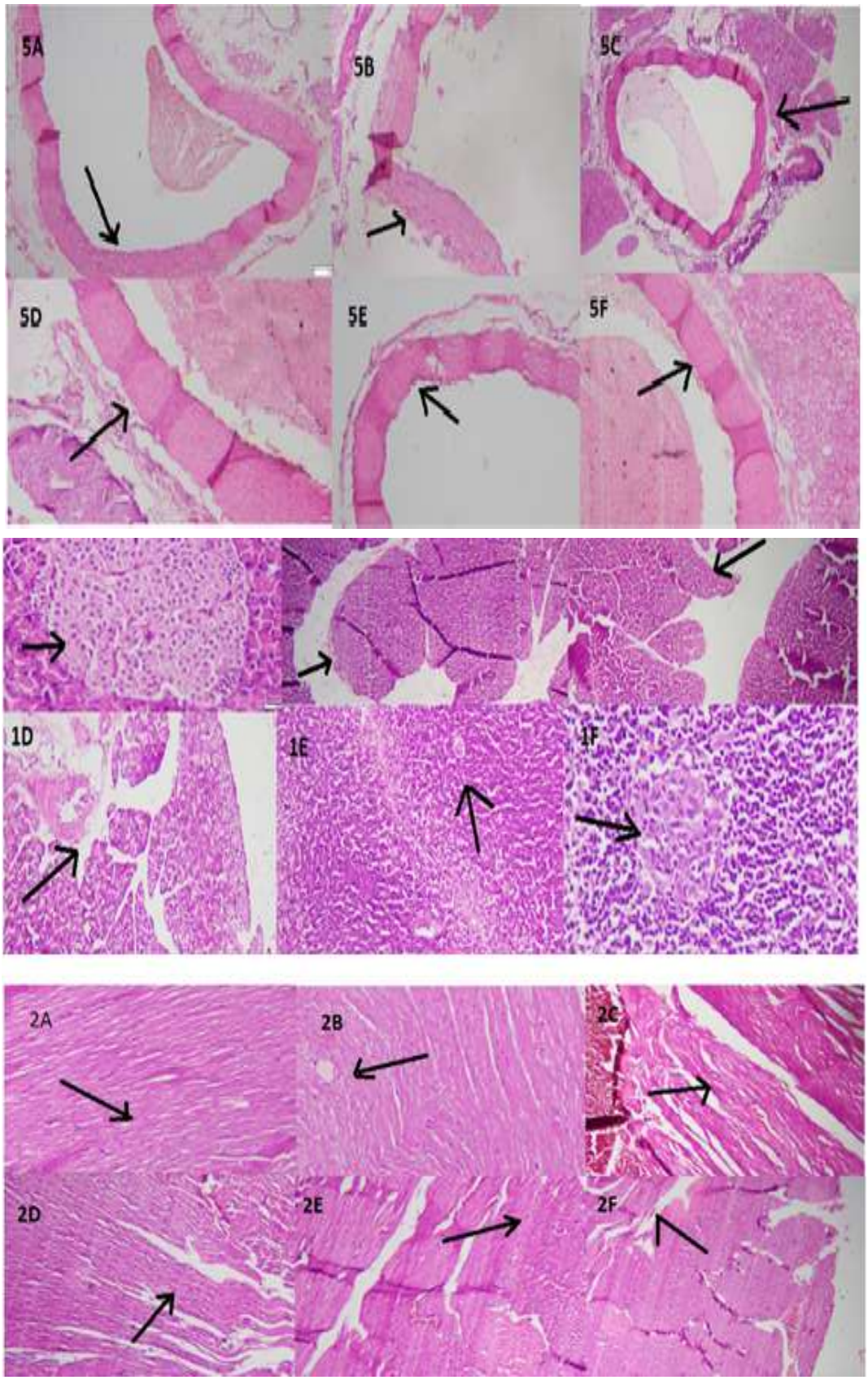

Fig. 4: All tissues staining done by hematoxylin and eosin.

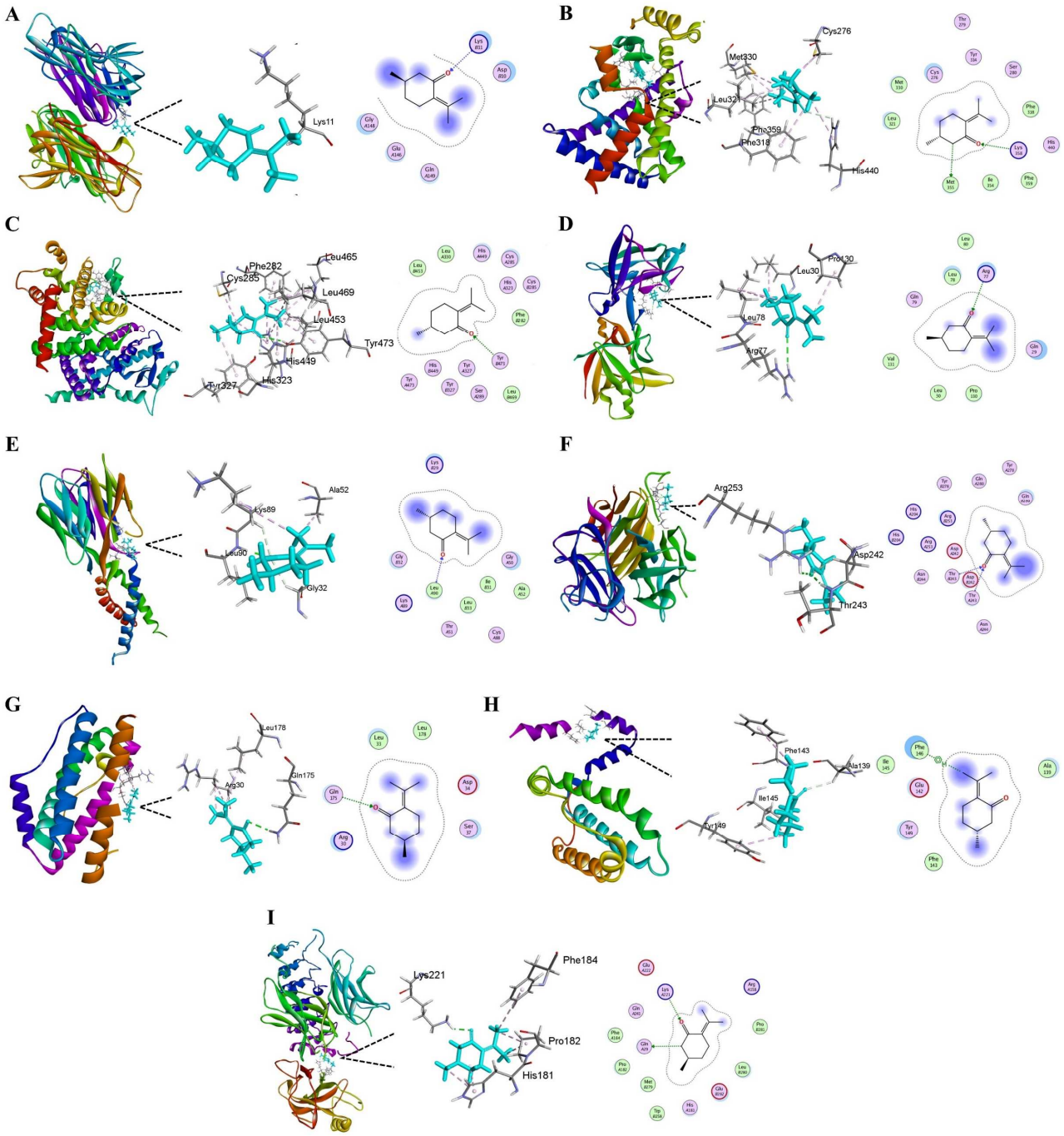

Fig. 5: Representation of molecular docking and posing analysis of pulegone with targeted proteins. 2D and 3D structures of pulegone with TNF-alpha (A), PPAR-α (B), PPAR-γ (C), IL-1Ra (D), Resistin (E), Adiponectin (F), IL-6 (G), IL-10 (H), NF-KB (I)

It was believed that interleukin served only as an immune system regulator (Al-Qahtani *et al.*, 2024). Despite its physiological role, this protein is also produced by other cells and is engaged in other physiological processes, according to research conducted so far. One naturally occurring cytokine that inhibits IL-1 is called IL-1Ra. The pro-inflammatory signal from the IL-1 receptor is halted when IL-1Ra attaches to the IL-1 receptor, blocking IL-1 binding (Bouayad, 2024). Research indicates that in both forms of diabetes, the anti-inflammatory IL-1Ra protects cell function while lowering the inflammatory effects of IL-1 (Guan *et al.*, 2024). Consequently, IL-1Ra may offer a novel treatment for type I and II diabetes mellitus (Luotola, 2022). Pulegone shows the strongest affinity for IL-1Ra, so together they effectively reverse insulin resistance.

The pro-inflammatory properties of IL-1 are competitive with those of IL-1 receptor antagonist (IL-1ra). Obesity affects adipokines, innate and adaptive immune cells, cytokine (interleukins) balance and expression levels and immune system function. Tumor necrosis factor (TNF), interleukin-6 (IL-6) and other immune system mediators were expressed differently, which led to inflammation, the advancement of obesity and the onset of related diseases, including diabetes(Memedi *et al.*, 2024). Moreover, it has been demonstrated that anti-inflammatory cytokines, such as interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1Ra), have important roles in the management of diabetes.

It has both pro- and anti-inflammatory properties and controls the immune response during hematopoiesis.

The pathophysiology of type 2 diabetes mellitus (T2DM) and the development of insulin resistance are both strongly influenced by the proinflammatory cytokine interleukin-6 (IL-6)(Ebrat *et al.*, 2024). IL-6 works biologically by binding to a membrane receptor that is made up of gp130 and two R-IL-6 subunits. Once these two receptors have attached to IL-6, they become soluble. Soluble gp 130 opposes the effects of IL-6, even though sR-IL-6 functions as an IL-6 agonist. In our study, the pro-inflammatory cytokine IL-6 levels were high in untreated rats, although its levels were significantly lower in treated rats.

Insulin resistance brought on by food is prevented by endogenous interleukin-10 (IL-10). Many cell types produce the pleiotropic cytokine IL-10. It mimics the activity of pulegone.

PPAR alpha, beta/delta and gamma-each have unique roles in metabolism. Energy homeostasis, oxidative stress, mitochondrial fatty acid metabolism and excitatory neurotransmission are all regulated by PPAR alpha; lipid metabolism, cell differentiation and myelination are regulated by PPAR beta/delta; and adipocyte differentiation, inflammation and mitochondrial biogenesis are regulated by PPAR gamma (Wawo *et al.*, 2024). It has also recently been discovered that PPARα contributes to the development of diabetic nephropathy (DN). Lipid accumulation and metabolism are closely linked to the progression of DN and PPARα deficiency appears to exacerbate the disease's severity by increasing the creation of extracellular matrix and inflaming the body (Nikpayam *et al.*, 2024). Heart fuel generation is dependent on mitochondrial FA β-oxidation, which is regulated by PPARα, which also controls lipid metabolism and cardiac energy. Anti-inflammatory PPARα in this study has higher levels in pulegone-treated rats as compared to untreated rats.

PPARG is recognized in two isoforms in humans and mice: PPAR-γ1 (found in almost all tissues except muscle) and PPAR-γ2 (found primarily in the intestine and adipose tissue). (PPAR-γ) is a ligand-dependent transcription factor that controls the metabolism of fat and glucose (Mohajan and Mohajan, 2024). It also inhibits angiogenesis and causes cell cycle arrest, which both contribute to apoptosis. Activation of PPAR-γ has demonstrated potential in vitro and numerous animal models of cancer (Chen *et al.*, 2024). Mature adipocytes that have activated PPARγ express more genes involved in the insulin signaling cascade, which enhances insulin sensitivity. Small, insulin-sensitive adipocytes are produced in response to stimulation by PPARγ, the master regulator of adipogenesis. So, in our work, PPARγ is released in higher quantities in treated rats in contrast to untreated rats.

Eukaryotic cells use NF-κB extensively as a regulator of genes that govern cell survival and proliferation (Zamanian *et al.*, 2024). Because NF-κB is misregulated in many distinct forms of human malignancies, it is constitutively active. It has been demonstrated that NF-κB plays a crucial role in the development of GLUT2, which helps β-cells secrete insulin in response to glucose. Therefore, inhibiting this transcription factor may have negative effects that result in the development of type 2 diabetes and insulin resistance(Yuan *et al.*, 2024).

Pulegone effectively weakened NF-κB in the current investigation, especially at larger doses. This attenuation may aid in lowering insulin resistance, indicating that pulegone may be a good option for diabetes treatment in the future.

Histological examinations showed significant pathological changes in diabetic rat tissues, including pancreatic islet cell loss, hepatocyte damage and kidney abnormalities induced by the high-fat, high-sucrose diet. Treatment with pulegone and metformin ameliorated these changes, indicating protective effects on various tissues, including the pancreas, liver, kidney, heart and aorta. The present study demonstrates that pulegone exerts significant anti-diabetic and anti-inflammatory effects, as evidenced by reductions in fasting blood glucose, HbA1c and pro-inflammatory cytokines (TNF-α, IL-6, resistin), alongside increased levels of adiponectin, IL-10, IL-1Ra and PPAR-γ.

The molecular docking results provide strong support for the *in-vivo* findings obtained through PCR and ELISA analyses. Pulegone demonstrated favorable binding affinities with several key proteins involved in inflammation and metabolic regulation, including IL-1Ra, PPARγ, NF-κB and IL-6. These interactions were characterized by stable hydrogen bonding and low RMSD values, indicating reliable docking poses. Notably, the high docking score with IL-1Ra correlates with the increased mRNA and protein expression observed *in-vivo*, suggesting that pulegone may enhance the anti-inflammatory activity of this receptor. Similarly, its strong interaction with PPARγ aligns with the upregulation of

PPARγ expression and improved insulin sensitivity seen in treated models. The binding of pulegone to NF-κB and IL-6 further supports its role in suppressing pro-inflammatory signaling, as reflected in the reduced cytokine levels measured by ELISA. Overall, the consistency between in silico docking scores and experimental gene/protein expression data reinforces the mechanistic plausibility of pulegone's dual anti-inflammatory and insulin-sensitizing effects.

These findings suggest that pulegone may modulate insulin signaling pathways through its influence on key molecular targets. Notably, the down regulation of NF-κB-a central mediator of inflammatory responses, may alleviate cytokine-induced insulin resistance, while the upregulation of PPAR-γ supports enhanced insulin sensitivity and lipid metabolism. The dose-dependent improvements observed across the 5, 10 and 15 mg/kg pulegone treatment groups further reinforce its therapeutic potential. However, while these results are promising, the pharmacokinetic profile of pulegone, including its bioavailability and potential off-target effects, remains to be elucidated. Future studies should investigate these aspects to determine whether pulegone can achieve effective concentrations *in-vivo* and translate into clinical applications. Overall, our findings provide mechanistic insight into pulegone's mode of action and establish a foundation for further exploration of its role in metabolic regulation.

CONCLUSION

Pulegone, a monoterpene ketone predominantly found in the essential oils of Lamiaceae mint species, demonstrated significant therapeutic potential in our study by alleviating insulin resistance and associated metabolic disturbances induced by a high-sucrose, high-fat diet in rats. It effectively downregulated pro-inflammatory markers (NF-κB, TNF-α, IL-6, resistin) and upregulated anti-inflammatory markers (adiponectin, IL-1RA, PPAR-α, PPAR-γ), contributing to improved metabolic profiles. Molecular docking analysis further supported pulegone's strong binding affinities with key inflammatory and metabolic receptors, reinforcing its potential role in modulating these pathways. These findings are promising, but they represent an early-stage investigation. Further research is essential to validate these effects in broader preclinical models, assess the compound's toxicity and pharmacokinetic properties and determine its ability to reach therapeutic concentrations *in-vivo*. Future studies should also explore its long-term safety, dose optimization and potential off-target effects. These steps are critical to establishing pulegone as a viable candidate for clinical development in the treatment of insulin resistance and related metabolic disorders.

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Authors' contribution
Conceptualization, original draft writing: Rubina Muhammad Ali, Aisha Mobashar, Abdullah Abdo Albajali. Reviewing: Tahir Maqbool, Kashif Barkat and Arham Shabbir data validation and editing: Khalid Hussain.

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Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval
This study was approved by the Research Ethics Committee of The University of Lahore (IREC-2022-26).

Conflict of interest
The authors declare that they have no conflict of interest regarding the publication of this research.

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