Metabolomics-guided exploration of *Pinus wallichiana* A.B. Jacks. cones for antidiabetic compounds: Integrating *in vitro*, *in silico*, *in vivo*, and histopathological approaches

Nigar Aksar[1], Muhammad Fiaz Khan[1]*, Mushtaq Ahmad Mir[2], Nasreena Bashir[2], Shumaila Noreen[1], Farhat Ullah[3], Abdul Sadiq[3], Muhammad Ayaz[3]*

[1]Department of Zoology, Hazara University Manshera, Dhodiyal Mansehra, KP, Pakistan; [2]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia; [3]Department of Pharmacy, Faculty of Biological Sciences, University of Malakand, Chakdara Dir (L), KP, Pakistan

***Corresponding Authors:** Muhammad Fiaz Khan, Department of Zoology, Hazara University Manshera, Dhodiyal Mansehra 21300, KP, Pakistan. Email: fiazkhanhu333@gmail.com; Muhammad Ayaz, Department of Pharmacy, Faculty of Biological Sciences, University of Malakand, Chakdara Dir (L) 18000, KP, Pakistan. Email: mayaz@uom.edu.pk

Academic Editor: Prof. Bernard Fioretti, University of Perugia, Italy

Received: 04 October 2025; Accepted: 18 December 2025; Published: 1 April 2026

ORIGINAL ARTICLE

Abstract

Pinus wallichiana A.B. Jacks., also known as Himalayan white pine, is recognized in traditional medicine for its various uses, including diabetes mellitus (DM). Pinus species like *Pinus roxburghii* (Chir pine) and *Pinus gerardiana* (Chilgoza pine) have shown promise for antidiabetic properties. This study focuses on the use of edible cones of *Pinus wallichiana (P. wallichiana)* for the potential management of DM. The methanolic extract of *Pinus wallichiana* (Pw.Cme) was subjected to Gas Chromatography–Mass Spectrometry (GC-MS) analysis, total phenolic content (TPC) and total flavonoid content (TFC) analyses, and qualitative phytochemical studies. The Pw.Cme and its derived fractions were evaluated for their *in vitro* antioxidant, α-glucosidase, α-amylase inhibitory studies, and the identified compounds were docked against enzyme targets, followed by Molecular Dynamic Simulation (MDS) studies. Detailed *in vivo* antidiabetic and histopathological studies were performed following standard procedures. The GC-MS analysis of Pw.Cme lead to identification of 45 compounds, and the *Pinus wallichiana* ethyl acetate (Pw.EtAc) fraction exhibited the highest TPC (258.55 mg gallic acid equivalent [GAE]/g) and TFC (63.05 mg quercetin equivalent [QE]/g). In 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays, the respective highest anti-radical activity was observed for Pw.EtAc, as IC_{50}=13.0 µg/mL and 5.3 µg/mL. In enzymes inhibition studies, considerable α-amylase inhibition was observed for Pw.EtAc with IC_{50}=1.98 µg/mL, and IC_{50}=7.2 µg/mL for α-glucosidase. *In vivo* studies indicated that the administration of Pw.EtAc resulted in a marked decrease in fasting blood glucose levels, hyperlipidemia, and weight loss in diabetic albino mice. Histopathology of vital organs of albino mice, administered with various doses of Pw.EtAc showed a healing effect against alloxan-induced lesions in the heart, pancreas, liver, and kidneys. In conclusion, we can claim that the extract of *P. wallichiana* cones is rich in many phytocomponents and have potential antidiabetic properties.

Keywords: diabetes mellitus; *Pinus wallichiana*; GC-MS; antioxidant; molecular docking

eISSN 2239-5687 online, DOI 10.15586/ijfs.v38i2.3394

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated glucose levels in blood plasma, resulting from insufficient secretion of insulin or inadequate action capability (Malviya, Jain *et al.,* 2010). Insulin, the hormone generated by the pancreas, controls how the body's cells absorb, use, and store glucose. Two main types of diabetes are type 1 diabetes mellitus (TIDM) and type 2 diabetes mellitus (TIIDM) (Kaul, Tarr *et al.,* 2013). In TIDM, the immune system targets and destroys the pancreatic beta cells responsible for insulin production, leaving the body completely insulin-deficient (Eisenbarth 1986). On the other hand, TIIDM is a disorder where the pancreas may either insufficiently produce insulin to satisfy the body's requirements or the body may develop resistance to insulin's effects (Edelman 1998). The long-term consequences of diabetes include nephropathy, retinopathy, and cardiovascular disorders (Sabir, Akhtar *et al.,* 2019). According to recent projections, the current global prevalence of diabetes (422 million) will increase to 622 million by 2040 (Safhi, Alam *et al.,* 2019). With millions of victims worldwide, DM is regarded as the most prevalent and fatal illness (Olokoba, Obateru *et al.,* 2012). Currently, several medications, including biguanides and sulfonylureas, are available for treating diabetic mellitus. To cure diabetes, it is important to look for a new class of molecules with more pronounced affects with less toxicity (Noor, Gunasekaran *et al.,* 2008). Alternative medications are being sought because of their more active role in diseases and less or no adverse effects.

The *Pinaceae* family indeed contains a large number of coniferous plants, with Pinus being the largest genus of this family. Almost 110 species distributed globally, Pinus is one of the most diverse and widespread genera of conifers (Little and Critchfield 1969). The needles of Pinus plant contain bioactive compounds, making them valuable as potential drug candidates for treating various health conditions (Singh, Dixit *et al.,* 2019). The Chilgoza Pine nut exhibited protective effect on oxidative stress and diabetes induced by streptozotocin in mice (*in vivo*). The extracts of pine nuts in methanol and ethyl acetate were evaluated for their antioxidant properties and the ability to inhibit α-amylase activity *in vitro*, and *in vivo* effects of various doses, which showed their therapeutic effect against diabetes (Zulfqar, Akhtar *et al.,* 2020). *P. wallichiana* A.B. Jacks. serve as a medicinal herb usually referred to kail or blue pine (Chaturvedi and Pandey 2001). Compounds such as phenolic and flavonoids were identified from the leaves and bark of *P. wallichiana* (Naeem, Taskeen *et al.,* 2010). The hydroalcoholic extract derived from both stem bark and leaves has a substantial *in vitro* activity against oxidative stress (Rahman, Uddin *et al.,* 2016). It has biological properties, which include antimicrobial, scavenging of free radicals, lowering of pro-inflammatory mediator, antipyretic, mutagenic, antipathogenic, antioxidant, antibacterial, antifungal, anticancer, and other ethnobotanical activities (Kumar 2015). The literature survey showed that no research has been conducted on *P. wallichiana* cones in the domain of investigation of biochemical composition as well as antioxidant and antidiabetic potential. Based on the ethnopharmacological relevance of *P. wallichiana* and other species in diabetes, the current study has been designed to assess the phytoconstituents, enzyme inhibitory, antioxidant, and anti-hyperglycemic role of extracts of *P. wallichiana* cones using *in vitro*, *in silico*, and *in vivo* approaches.

Methodology

Chemical and drugs

Alpha-amylase MACKLIN, Lot No. C15997347, Shanghai Macklin, China, Starch CAS 9005-25, Shanghai Macklin, China, α-glucosidase CAS 9001-42-7, Shanghai Macklin, China, Glucopyranoside (4-Nitrophenyl α-D-glucopyranoside) CAS 3767-28-0, Sigma-Aldrich, USA, Acarbose ALFA AESAR, Cat No. J61737.

Plant collection and processing

The cones of *Pinus wallichiana* A.B. Jacks were obtained from Malamjabba, Swat, Khyber Pakhtunkhwa, Pakistan and authenticated by Dr. Azhar Khan, a botanist at the Department of Botany, Hazara University, Manshera, Pakistan. The cones were dried in shade and submitted for authentication under voucher No. Z.HU.PHD.022 to the herbarium of the Department of Botany, Hazara University, Pakistan.

Extraction

To remove pollutants, soil, and dust particles, the plucked cones were cleaned with a blower and washed with distilled water. All the cones were kept in a dark room for 45 days for drying at 25 ± 2°C and 55 ± 5 Relative Humidity (RH) under controlled conditions. The dried cones were crushed and sieved through a 60-mesh stainless steel sieve before being extracted and kept in an airtight flask for usage. The powdered cones were placed in a stainless steel container and soaked in 80% methanol for 10–14 days, during which the mixture was shaken occasionally (Zulfqar, Akhtar *et al.,* 2020). After the soaking period, the solvent (methanol with dissolved phytochemicals) was separated from the cone material. It was first sieved through a gauze cloth, and then the solution was further filtered by passing through a filter paper. The separated filtrate was evaporated using rotary evaporator at 40°C under low pressure. Subsequent to the

evaporation process, a red-brown semisolid *Pinus wallichiana* methanolic extract (Pw.Cme) was acquired. The Pw.Cme was further used for fractionation.

Fractionation

Solvent–solvent extraction was performed to acquire various fractions from Pw.Cme based on increased polarity. The powdered cones were soaked in 500 mL of methanol, n-hexane, ethyl acetate, and chloroform. Each fraction was subsequently agitated for 72 h in a shaking incubator maintained at room temperature, operating at a speed of 100 revolutions per minute (rpm). Then under specific vacuum conditions, methanol, n-hexane, ethyl acetate, and chloroform fractions were sieve-evaporated using a rotary evaporator. Finally, until future usage, all cone fractions were preserved at 4°C in a refrigerator.

Phytochemistry

Gas Chromatography–Mass Spectrometry (GC-MS) analysis

To identify compounds in Pw.Cme, GC-MS analysis was conducted using GC-MS analyzer (Clarius 500 Perkin Elmer, PerkinElmer, Inc., Waltham MA, USA). The Elite-1 fused silica, 30 m (length) × 0.25 mm (ID), 0.25 µm (thickness), 100% (dimethyl polysiloxane) was used. The analysis was performed in electron impact mode at 70 eV. An injection volume of 1 µL was employed with a split ratio of 10:1. Helium (99.999%) served as a carrier gas, maintaining a continuous flow rate of 1.51 mL/min. Temperatures of injector and ion source were maintained at 240°C and 200°C, respectively. The first-column temperature was retained at 45°C for 2 min (isothermal) before being programmed to 300°C at a rate of 5°C/min for 5 min (isothermal). The temperature of the injector was 250°C. The flow rate of helium carrier gas was 1.41 mL/min. The following parameters were used to obtain all of the mass spectra: equipment current: 60 mA; filament emission current: 70 eV; ionization voltage: 200°C; and ion-source split mode used to inject diluted samples: 1% v/v, at a split ratio of 1:15. The recognition of constituents was performed based on factors, such as molecular structure, mass, and calculated fragments. Molecular mass and structure provide important clues about the identity of compounds in the extract.

To align the mass spectrum data with recognized patterns, the mass spectrum analysis data were assessed in relation to the National Institute of Standards and Technology (NIST) database, Main Library Alliance (MAINLIB), and REPLIB libraries. A large collection of mass spectra patterns from different compounds are found in the NIST database. It has over 62,000 patterns. The compounds in the cone's extract were recognized by name, chemical structure, and molecular weight by using the NIST database. The spectrum of the unknown component was evaluated against the spectrum of a known compound or compounds archived in the NIST (2005) software, Turbomas 5.2.

Qualitative phytochemical analysis

Below are the protocols followed for the qualitative detection of phytochemicals in Pw.Cme of *P. wallichiana* cones and its derived fractions.

Reducing sugars

Reducing sugars in the extracts were detected by Benedict's test described by Kodangala and colleagues (2010). The extract samples were heated for 5 min in a water bath. Following the heating process, 0.5 mL of Benedict's reagent was added in the extract. Formation of a brick red precipitate after the addition of Benedict's reagent showed the presence of reducing sugars in the sample. To confirm the reliability of the test, dextrose was used as a positive control (Kodangala, Saha *et al.,* 2010).

Amino acids

The assay for identifying amino acids in the cones' extract involved mixing 0.5 mL of cones extract in a solution of ninhydrin (3 drops). The solution was heated to boiling in a water bath for about 10 min. Appearance of a purple color during or after the heating process showed the presence of amino acids in the sample. To ensure reliability of the test, glycine (a known amino acid) was used as a control (Ahuja, Suresh *et al.,* 2011).

Lignin

The method reported by Linga Rao and Savitharamma (2011) was used with slightly modifications. A 500 µL of extract was mixed with 500 µL of gallic acid. The presence of lignin was indicated by olive green color.

Leucoanthocyanins

For the detection of leucoanthocyanins the cones extract sample, 400 µL, was combined with 400-µL isoamyl alcohol. Red coloration in the upper layer showed the presence of leucoanthocyanins.

Steroids

For detection of steroids, a sample of 0.5 mL was taken and 1 mL concentrated sulfuric acid (H_2SO_4) was incorporated. It was shaken well and left aside for 2 min; appearance of reddish-brown color indicated the presence of steroids.

Terpenoids

The terpenoids detection method was described by Singh and coworkers (2019) with few modifications

(Pradeep, Dinesh *et al.,* 2014). Samples extracted from the cones were mixed with 2 mL of chloroform. Then few drops of concentrated H_2SO_4 were added. The appearance of a reddish-brown color at the interface indicated the presence of terpenoids.

Tannins

Concentrated hydrochloric acid (HCl), 100 µL, was mixed with 500 µL of sample solution of the extract, followed by heating for 10 min. The emergence of red color precipitate indicated the presence of tannins (Akinjogunla, Yah *et al.,* 2010).

Quantification of Total flavonoid content (TFC) and Total phenolic content (TPC)

For determining flavonoid and phenolic contents in cone extracts, aluminum chloride colorimetric and Folin–Ciocalteu (F-C) methods were used (Wan Mohd Zain, Ramli *et al.,* 2021). About 100 µL of the extract was taken and 10 mL of methanol was added in a Falcon tube. The solution mixture comprising of 50 µL of 1 Molar potassium acetate, 50 µL of 10% aluminium chloride, 300 µL of methanol and 760 µL of distilled water were combined and thoroughly mixed. For preparing sample blanks, distilled water was used instead of aluminium chloride; 10 mg of quercetin was added in methanol to make dilutions. A calibration curve was made with quercetin and optical density (OD) was measure at 415 nm. The TPC was assessed using the Folin–Ciocalteu method with modest variations (Wan Mohd Zain *et al.*, 2021). To begin, 100 µL of cone extract was prepared, followed by incorporation of distilled water (540 µL) and Folin–Ciocalteu reagent (60 µL). The mixture was placed at 37°C for incubation for about 5 min. Finally, 600 µL of 7% sodium carbonate (Na_2CO_3 in deionized water) solution was mixed in the sample solution and placed at room temperature for 90 min to incubate. The OD of both sample and reference solutions, along with blank reagent, was measured at 550 nm using the UV-Visible spectrophotometer. All tests were conducted in triplicate. The results were reported as mg of gallic acid equivalent (GAE)/g of the sample extract and mg of quercetin equivalent (QE) per gram of the sample.

Antioxidant studies

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was carried out following previously reported approach with some modifications (Nawaz, Sadiq *et al.* 2025). DPPH solution was made by dissolving 24-mg DPPH in methanol (100 mL). Test sample solutions of various concentrations (62.50–1,000 µg/mL) were prepared. From each dilution, 1-mL sample solution was taken, to which 1 mL DPPH solution was added. The solution mixture was incubated in the dark at 23°C for 30 min and then subjected to UV-Vis spectrophotometer to note absorbance at 517 nm. The absorbance values of all samples were taken in triplicate. Ascorbic acid served as a positive control, while negative control contained only DPPH solution having no test samples. The experiment was performed in triplicate and DPPH %inhibition was calculated (Wan Mohd Zain, Ramli *et al.* 2021).

2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay

Antioxidant activity was carried out using ABTS free radicals (Vinci, D'Ascenzo *et al.* 2022). ABTS (7 mM) along with potassium persulfate (2.45 mM) were prepared and subsequently mixed and kept in low beam light for 12–16 h for formation of free radicals. After incubation through addition of methanol (50%) absorption was adjusted in the range of 0.7 at 745 nm. Then 300 µL of plant extract was mixed with 3.0 mL of ABTS solution in a cuvette. After 6 min, absorbance was measured through double beam spectrophotometer. Ascorbic acid was used as a positive control. Results were taken in triplicate. %ABTS scavenging activity was measured by using the following formula:

$$\text{Scavenging impact (\%)} = \left[\frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}}\right] \times 100$$

In vitro anti-diabetic studies

α-Amylase inhibition

The cone fraction extracts were investigated for α-amylase inhibition by utilizing the technique of 3,5-dinitrosalicylic acid (DNSA), with acarbose serving as a standard with some modification as reported by Zulfqar *et al.* (2020). Saline phosphate buffer was used as a negative control. The solution was prepared by mixing 1 mL of cone's fraction extract with 1 mL of α-amylase enzyme (porcine pancreatic amylase solution, 0.1 mg/mL) and incubated for 30 min at 25°C. After incubation, 1 mL of starch solution was added to individual tube; 1% starch solution was used as a substrate for α-amylase. Thereafter, one milliliter of 3,5-dinitrosalicylic acid (DNSA) reagent was added to the test tubes and heated in a water bath for 5 min at 90°C. After heating, the test tubes were chilled to 25°C. To make the final volume 10 mL, distilled water was added to the reacting solution. Using a spectrophotometer, the OD of each sample was measured at 540 nm and the percentage inhibition of α-amylase was identified.

α-Glucosidase inhibition

The method involves the use of acarbose as a reference for comparison and the measurement of IC_{50}

values. The α-glucosidase (saccharomyces cerevisiae, recombinant) solution was prepared at a concentration of 0.5 units/mL in phosphate buffer of 0.1 M, pH 6.90. In buffer solution, a substrate solution of 5 mM of p-Nitrophenyl-α-D-glucopyranoside was incorporated. The α-glucosidase solution was mixed with sample solutions at specified concentrations and placed for incubation at 37°C for 15 min. The solution of substrate was mixed, and further incubation was conducted. The final reaction was completed by adding a solution of sodium carbonate (0.2 M, 80 µL). Absorbance of solutions (including the test samples/blank) were recorded at 405 nm (Mahnashi, Alqahtani *et al.* 2021).

In silico analysis

Ligand preparation

In order to prepare ligands, we sourced the 3D structures of identified compounds during GC-MS analysis. Some of the ligands' 2D structures were drawn manually using ChemDraw 3D 15 (Hanwell, Curtis *et al.* 2012).

Preparation of protein

To prepare proteins we utilized the Data Bank of Protein (https;//www.rcsb.org). The Discovery Studio Visualizer (version 20.1.0.19295) was employed to fresh proteins and incorporate H_2 atoms. Energy optimization was performed by the process using Swiss PDB Viewer (Guex and Peitsch 1997).

Molecular docking and visualization

For visualization and docking, we selected residues of amino acid from the active site and conducted the docking by using Autodock vina (Guex and Peitsch 1997). To analyze the results, we utilized Ligplot plus version 2.2.4 (Dallakyan and Olson 2015).

In vivo anti-diabetic studies

From *in vitro* study, we concluded that ethyl acetate had shown better antidiabetic activity as compared to other fractions ($p < 0.05$). Therefore, *in vivo* study was conducted to evaluate the role of *Pinus wallichiana* Pw.EtAc fraction against diabetes in animals.

Experimental animals and ethical approval

Albino mice of either gender weighing 230–270 g were obtained. The animals were kept in cages of animal house and acclimatization to laboratory conditions (25±2°C, and 12 h dark and light cycle) was carried out along with the provision of proper diet and water *ad libitum* for 2 weeks. However, diabetic animal's diets were withheld for a whole night to measure their fasting blood glucose level. Both male and female mice were kept in separate cages. The trial was approved by the Animal Ethical Research Committee (Ref. No. 055). The care and treatment of trial animals was conducted in accordance with the National Institute of Health (NIH) regulations.

Group I: Normal control (nondiabetic), received normal saline at 5 mL/kg/day

Group II: Diabetic control (disease), received alloxan at 160 mg/kg, single dose

Group III: Standard control, administered with acarbose at 5 mg/kg/day)

Group IV: Treated with Pw.EtAc at 150 mg/kg/day

Group V: Treated with Pw.EtAc at 300 mg/kg/day

Acute toxicity studies

Mice were used to evaluate the toxic level of ethyl acetate fraction. Mice were given oral dose of 3,000 mg/kg of the extract and were closely monitored for 30 min initially and then at 4, 12, 24, and 48 h. Any sign of toxicity, change in behavior, and mortality was noted during this period (Sadiq, Zeb *et al.* 2018).

Induction and assessment of diabetes

Alloxan monohydrate (160 mg/kg) was freshly prepared in a citrate buffer. Male/female albino mice fasted overnight were injected with alloxan monohydrate via intraperitoneal (i/p) route. Glucose was given as solution at a dose of 2 g/kg to prevent alloxan-induced abrupt hypoglycemia. The mice were observed for the following 3 days. After 72 h of administration, blood glucose levels were measured by collecting blood from the tail vein. A glucometer (ACCU-CHECK Performa, Germany) was used to measure blood glucose level (Ngugi, Kimuni *et al.* 2015). For the experimental study, mice with serum glucose level >300 mg/dL were taken. Ethyl acetate fraction was administered orally. On day 28, vital organs were taken from animals administered with ethyl acetate extracts and changes in organ weights were assessed and compared with normal and standard control.

Biochemical analysis of blood samples

After 28 days, blood from each animal was collected through cardiac puncture and animals were sacrificed to collect organs for histopathology. Various blood tests

were conducted to assess the level of following biomarkers in blood: levels of lipids (e.g., cholesterol and triglyceride), parameters for functioning of the kidneys, such as creatinine and blood urea, liver function tests (LFTs), such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin.

Lipid profile

For measuring lipid profile, that is cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) in a serum sample, a 10-mL serum sample was mixed with a triglyceride solution (1,000 mL) and incubated at 37°C for 10 min. Absorbance at 546 nm was selected to quantify triglyceride concentration in serum sample. With the use of diagnostic kits, 1,000 µL of cholesterol solution and 10 mL of serum sample solution were combined and placed for incubation for 10 min at 37°C. The absorbance was recorded at 546 nm in comparison to a blank (control sample that had no serum). The absorbance reading indicated the total cholesterol concentration in serum sample. About 200-µL serum was mixed with HDL solution (500 µL). The solution was permitted to stand for 5 min at room temperature. The solution was mixed and subjected to centrifugation for 5 min. Centrifugation separates HDL from other components in the sample, and the supernatant (liquid above the solid pellet) was collected. The cholesterol solution (500 µL) was mixed with HDL supernatant (50 µL) and incubated for five min at 37°C. The sample's OD was calculated at 546 nm. The quantity of LDL was measured by using the following formula:

$$\text{LDL} = \text{Total Cholesterol} + \text{High density lipoprotein} - \frac{\text{Triglycerides}}{5}$$

Renal functions test (RFT)

Procedure for determining renal function using biochemical tests, particularly for measuring uric acid, creatinine, and blood urea, are commonly used to assess kidney function and diagnose renal condition. A 500 µL of reagent was mixed with about 50 µL of serum solution and the mixed solution was placed for incubation at 37°C for 1 min. Using a spectrophotometer, the absorbance of the solution was measured at 500 nm. For measuring blood urea, serum sample (10 µL) and 1,000 µL of enzyme reagent-1 were mixed and incubated for 5 min at 25°C. Reagent-2 of 1,000 µL was incorporated after incubation. The absorbance was measured at 578 nm using a spectrophotometer.

Liver function tests

Liver function tests were used for verifying liver biomarkers, specifically alkaline phosphatase, serum bilirubin, and serum glutamate pyruvate transaminase levels. For measuring ALT, serum sample, 50 µL, was mixed with reagents R1 and R2 (400 µL and 100 µL). The sample was incubated for 30 s at 37°C and OD was measured at 340 nm. For ALP test, 10 µL of serum sample was mixed with reagents R1 (400 µL) and R2 (100 µL). The mixture was incubated for 30 s at 37°C. For bilirubin, reagent R1 (100 µL) was mixed with reagent R2 (25 µL), and 100 µL of serum sample was added to this mixture. Reagent R3 (500 µL) was mixed to the above solution and allowed to stand at 25°C for 5 min. Finally, 500 µL of R4 reagent was added to the mixture and incubated for an additional 5 min at 25°C. After 5 min, the absorbance was measured at 546 nm through a spectrophotometer.

Histopathological analysis

Albino mice were dissected to access and remove specific organs, such as the heart, liver, kidney, and pancreas. After removal, the dissected organs were well preserved at room temperature in 10% formalin for 24 h. Histology staining procedure involved several key steps: Firstly, tissue processing, including fixation, embedding, and sectioning. Secondly, the staining process using hematoxylin and eosin (H&E) to highlight different cellular components. Thirdly, removal of paraffin, rehydration, staining with hematoxylin, "bluing," and staining with eosin. Finally, dehydration, clearing, and mounting on a slide for microscopic examination.

The slide was immersed in hematoxylin to stain the nuclei blue. Excess hematoxylin was removed, often with an acid-alcohol solution, to clarify the nuclear detail and rinsed in water or a bluing agent to provide the nuclei a deeper and more permanent blue color. The slide was stained with eosin, which stained the cytoplasm and other components pink and passed through increasing concentrations of alcohol to dehydrate the tissue, followed by a clearing agent like xylene. A mounting medium and a coverslip were applied to protect the slide and allowed for microscopic viewing. A bar scale of 20 µm × 5 µm with 40× magnification was set to view images and take photographs. The below blinded scoring (4-point) was followed for the analysis of histology images.

Data analysis

All the values obtained from the experiments, including biochemical parameters and IC_{50} values were presented as mean ± SEM. Tukey's multiple comparison test was employed to perform one-way analysis of variance (ANOVA) for biochemical parameters. The significance level was set at $p \leq 0.05$. The IC_{50} values were calculated using Microsoft Excel.

Results

Identification of biomolecules by GC-MS analysis

The GC-MS study of crude methanolic extract of cones of *P. wallichiana* discovered the existence of 45 different compounds. The GC-MS spectrum is shown in Figure 1. The identified compounds' retention duration using the provided GC technique ranged from 3.39 min to 27.37 min. Table S1 of the supporting information (GC-MS analysis of cones of *P. wallichiana* crude methanolic extract) lists all identified compounds' specifications. As a percentage of retention time, the chromatogram displays the relative concentrations of various components that were eluted. The relative concentrations of chemicals that exist in Pw.Cme were indicated by the height of various peaks. The chemicals that were eluted at various periods were subjected to mass spectrometer analysis to determine their type and structure. These spectrums serve as the compound's fingerprints, which the library can identify. The identification of potential chemical ingredients may necessitate qualitative examination of chemical substances. An important technique for this research is the combination of mass spectrometry with gas chromatography and this is characterized by a mass spectrum and offers qualitative information about chemical constituents. The bioactive components in the crude extracts of the cones were identified using their RT, molecular weight, and formula, peak area (%). *P. wallichiana* cones were analyzed for first time and 45 compounds were discovered that could influence the pharmaceutical properties of the plant.

The first compound with less retention time of 3.39 was Urs-12-ene, whereas the last compound having retention time of 27.37 was 9,12,15-octadecatrienoic acid as shown in Table S1 (GC-MS analysis of cones of *P. wallichiana* crude methanolic extract).

Qualitative phytochemical analysis

Table 1 refers to the qualitative testing of phytochemicals in extracts derived from *P. wallichiana* cones. The potential applications of these phytochemicals in various sectors, such as medicine, cosmetics, and food, make them a subject of interest. All extracts from *P. wallichiana* cones exhibited high levels of phenols, tannins, and flavonoids. These substances are recognized for their antioxidant characteristics. Benedict's test revealed high concentration of carbohydrates in ethyl acetate extract as compared to the methanol extract. None of the *P. wallichiana* cone extracts contained amino acids. Labat's and Dalhman's tests showed the existence of lignin in all extracts. The appearance of yellow and olive-green colors indicated this. The glycoside content found in methanolic extract was much higher than that of the ethyl acetate fraction. When sulfuric acid was tested, a dark reddish-brown color was seen, indicating this distinction. Leucoanthocyanins, which give extracts their deeper red color, were present in certain extracts. Nevertheless, the ethyl acetate extract lacked them. All the extracts contained terpenoids, although the ethyl acetate extract had the highest concentration of terpenoids. The interface's maximum reddish-brown color served as an indicator of this.

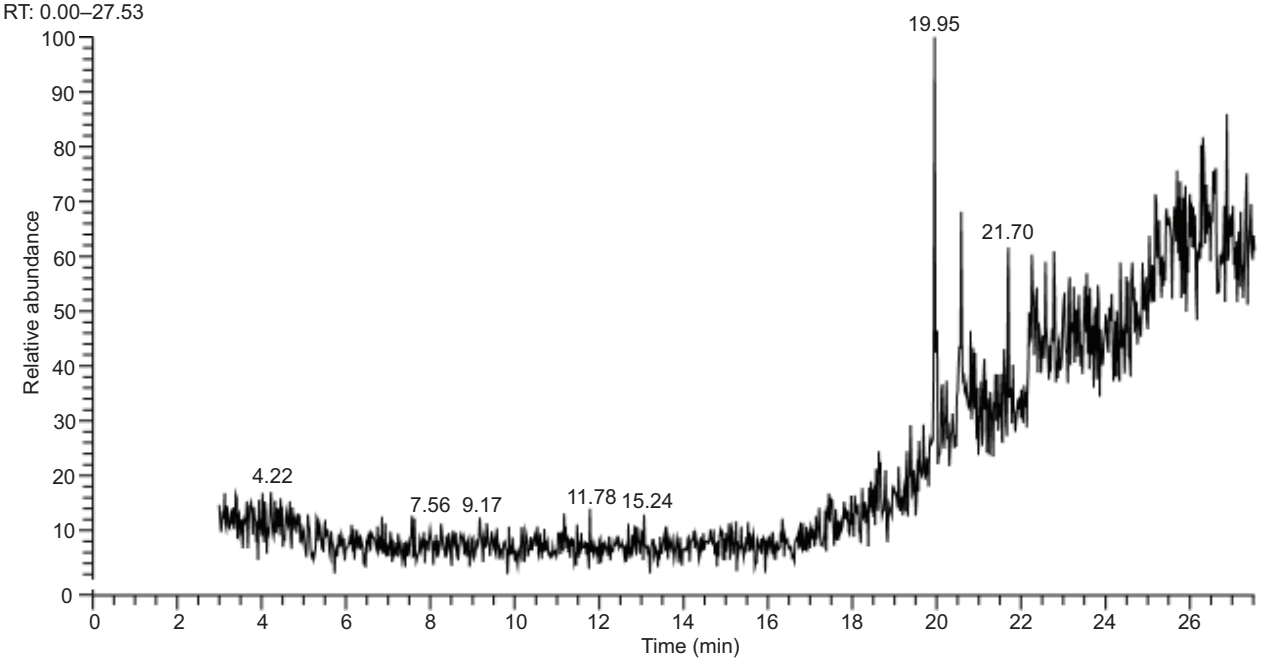

Figure 1. GC-MS chromatogram of the cones of *P. wallichiana* Pw.Cme.

Table 1. Phytochemical analysis of cones of *P. wallichiana.*

Test	Pw.Cme	Pw.Hex	Pw.Chf	Pw.EtAc
Reducing sugar	+	+	+	+
Amino acids	–	–	–	–
Lignin	+	+	+	+
Steriods	+	+	+	–
Leucoanthocyanins	+	+	+	–
Terpenoid	+	+	+	+
Tannin	+	–	+	+

Note: Present (+), absent (-) *Pinus wallichiana* Pw.EtAc.

Table 2. Quantitative phytochemical analysis of fraction extracts of *P. wallichiana* cones.

Plant species	Extracts	TFC (mg QE/g dry weight)	TPC (mg GAE/g dry weight)
Pinus wallichiana	Pw.EtAc	63.05±0.08^{ns}	58.55±0.03^{ns}
	Pw.Cme	55.77±0.01***	51.22±0.03^{ns}
	Pw.Chf	44.48±0.01***	43.69±0.02**
	Pw.Hex	39.24±0.00***	35.23±0.04**

Note: Data were presented as mean ± SEM; TPC: total phenolic content; TFC: total flavonoid content; *Pinus wallichiana* Pw.EtAc.
ns: Not significantly different when compared with control.
**: $p < 0.01$ and ***: $p < 0.001$.

Table 3. DPPH and ABTS IC_{50} (µg/mL) values and standard ascorbic acid.

Cones fraction	$IC_{50/DPPH}$ (µg/mL)	$IC_{50/ABTS}$ (µg/mL)
Pw.EtAc	13.0±0.05	5.3±0.07
Pw.Chf	95±0.06	18±0.03
Pw.Cme	150±0.03	250±0.08
Pw.Hex	402±0.02	232±0.02
Ascorbic acid	10±0.07	15±1.3

Note: *Pinus wallichiana* Pw.EtAc.

Quantitative Analysis of Total Phenolic and Total Flavonoid contents

Compared to the methanolic, chloroform, and n-hexane extracts, which were approximately 51.22±0.03, 43.69±0.02, and 35.23±0.04 mg GAE/g, Pw.EtAc cones extract (58.55±0.03) had a higher TPC. The TPC was measured by absorbance at 765 nm by using a calibration curve for gallic acid with the equation y=0.0014x + 0.0784 (R^2=0.9963), where 'x' represents TPC and 'y' represents absorbance. The absorbance of various concentrations was used to evaluate TFC in cone's fractions, and the standard calibration curve was constructed to calculate TFC. The highest flavonoid's content was present in ethyl acetate fraction 63.05±0.08 mg QE/g, compares to methanol, chloroform, and n-hexane, which were about 55.77±0.01, 44.48±0.01, and 39.24±0.00 mg QE/g (equivalent of quercetin). The cone fraction's TFC concentrations were estimated using the equation y=0.0023x+0.1849 (R^2=0.9471), which was based on the standard calibration curve for QE and absorbance at 415 nm as shown in Table 2.

DPPH and ABTS radical scavenging results

The DDPH and ABTS results are shown in Table 3. The DPPH method was used to assess the antioxidant capacity of the cones of *P. wallichiana* n-hexane, chloroform, methanol, and ethyl acetate fractions *in vitro*. Ascorbic acid was used as a positive control. Three tests were conducted for each sample. However, the DPPH was highly inhibited by the ethyl acetate extract (63.85±0.05) compared to the chloroform extract (60.90±0.06) at 500 mg/mL. The level of antioxidant potential necessary to lower the concentration of the radical (DPPH) by 50% was identified using IC_{50} values, which were used to evaluate the analyzed sample's capacity to inhibit DPPH. These values were inversely related to antioxidant activities. At different concentrations, fractions of cones of *P. wallichian* achieved 90.82±0.07%, 88.16±0.03%, 54.93±0.02%, and 57.84±0.08% inhibitions in the ABTS test. *P. wallichian's* Pw.EtAc fraction had an estimated IC_{50} of 5.3 µg/mL for scavenging ABTS free radicals. Ascorbic acid showed 92.21% inhibition with an IC_{50} value of 15 µg/mL.

α-Amylase and α-glucosidase inhibition assay

The four *P. wallichiana* cone fractions were subjected to an *in vitro* experiment of α-amylase and α-glucosidase inhibitory activity using starch as a substrate and acarbose as a positive control whose results are shown in Figure 2. Extracts of ethyl acetate did not significantly vary from acarbose in their ability to inhibit α-amylase. However, at the same doses, both methanolic and n-hexane extracts displayed greater α-amylase activity than the chloroform extract. Ethyl acetate (79.55± 0.49) and methanolic (65.02± 3.31) extracts exhibited a prominent inhibition of α-amylase.

Molecular docking studies

The compounds obtained from GC-MS were subjected to docking in the binding sites of α-amylase and α-glucosidase. We utilized docking as a tool to investigate

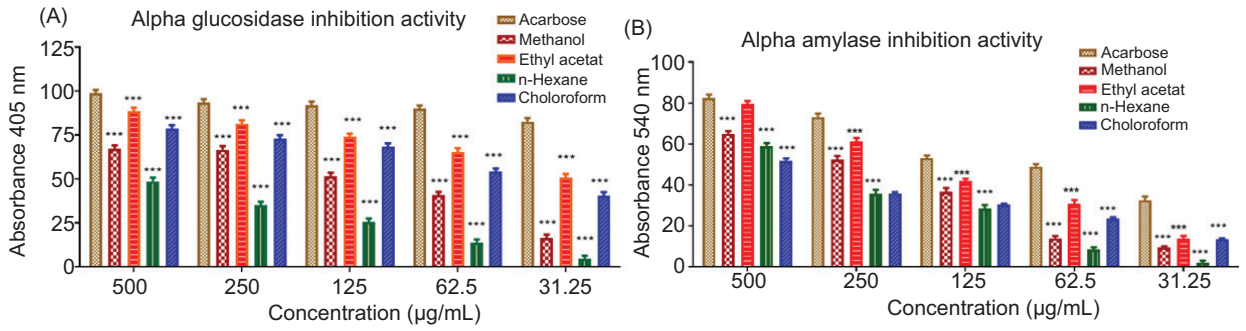

Figure 2. Inhibition of (A) α-glucosidase and (B) α-amylase activity at 500 µg/mL. The fraction at 500 µg/mL exhibited 88.56% inhibition. At 250, 125, 62.5, and 31.25 µg/mL, the response of these concentrations was also seen, that is 81.52%, 73.93%, 65.43%, and 60.90%. The standard acarbose IC_{50} value was recorded as 2.0 µg/mL. As compared to methanol and n-hexane fraction, chloroform extract showed the highest %inhibition.

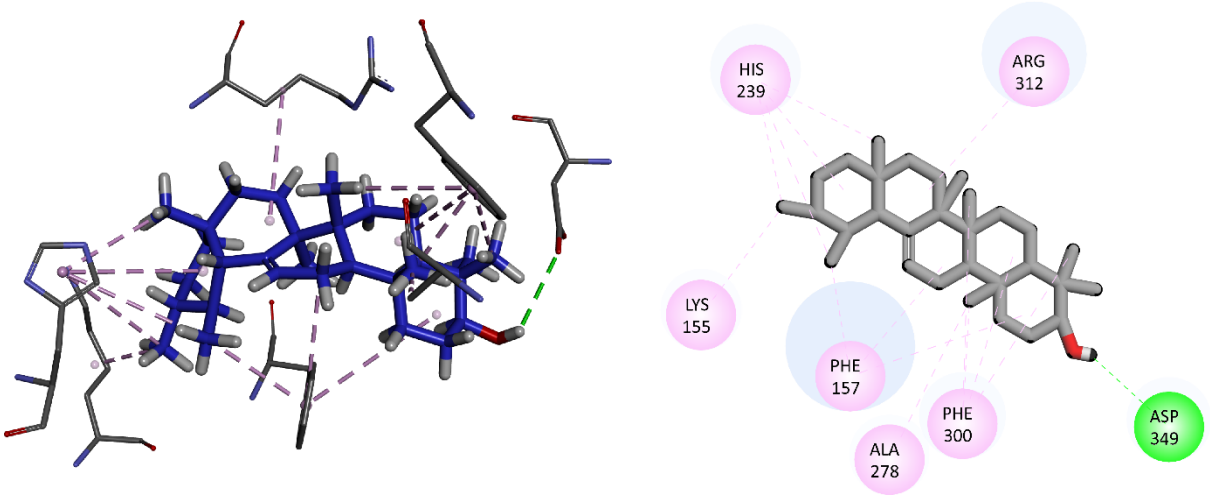

Figure 3. 2-D/3-D interaction plot of α-amyrin into the binding site of homology modeled α-glucosidase.

changes in binding orientation and binding energy of cones of *P. wallichiana* compounds. To achieve this, we utilized the homology-modeled of α-glucosidase that was formerly presented by our research team for docking (Hussain, Khan *et al.* 2019). Additionally, we acquired 3D crystal structures of α-amylase from Prodtein Data Bank (PDB) with accession number 4W93.

The interaction plots depicting the behavior of α-amyrin are shown in Figure 3. It establishes hydrogen bond interactions with Asp349. Moreover, aside from hydrogen bond interactions, Lys155, Phe157, His239, Ala278, Phe300, and Arg312 show pi-alkyl, alkyl interaction with a carbon atom of main structure. The computed binding energy value was -6.5857 kcal/mol. The interaction plots of androst-5-en-17-one and 3-methoxy-,17-methoximeare shown in Figure 4. His279 and Phe300 form interaction with π-Alkyl. It forms hydrogen bond interactions with Glu304, His239, Asp408, Phe157, and Arg312.

The interaction plots of α-amyrin are shown in Figure 5. It forms pi-alkyl hydrophobic interaction with Tyr151, Tyr62, Trp58, Leu162, His201, Ala307, and Ile235. The computed binding energy was -6.4323 kcal/mol. In 3,7,11-trimethyl-dodeca-2,4,6,10-tetrae nal forms hydrogen bond interactions with Arg439 and Asp68. Phe177, His279, and His239 interact with π-Alkyl. The computed binding energy value was -7.8986 kcal/mol.

The 3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-2-yl) icosahydro-1H-cyclopenta[a]chrysen-9-ol (Lupeol) forms hydrogen bond interactions with Glu A:233. His A:305, Tyr A:62, and Trp A:59 form interactions with π-Alkyl. The N-(2-((2-(benzylamino)-2-oxoethyl)thio)-1,3-benzothiazol-6-yl)-4-bromobenz amide form hydrogen bond interaction with Asp A:197 and His A:299. Trp A:59 interact with π-Alkyl. The 3,7,11-trimethyl-dodeca-2,4,6,10-tetrae nal formed one hydrogen bond interaction with His A:299; His A:201 formed alkyl

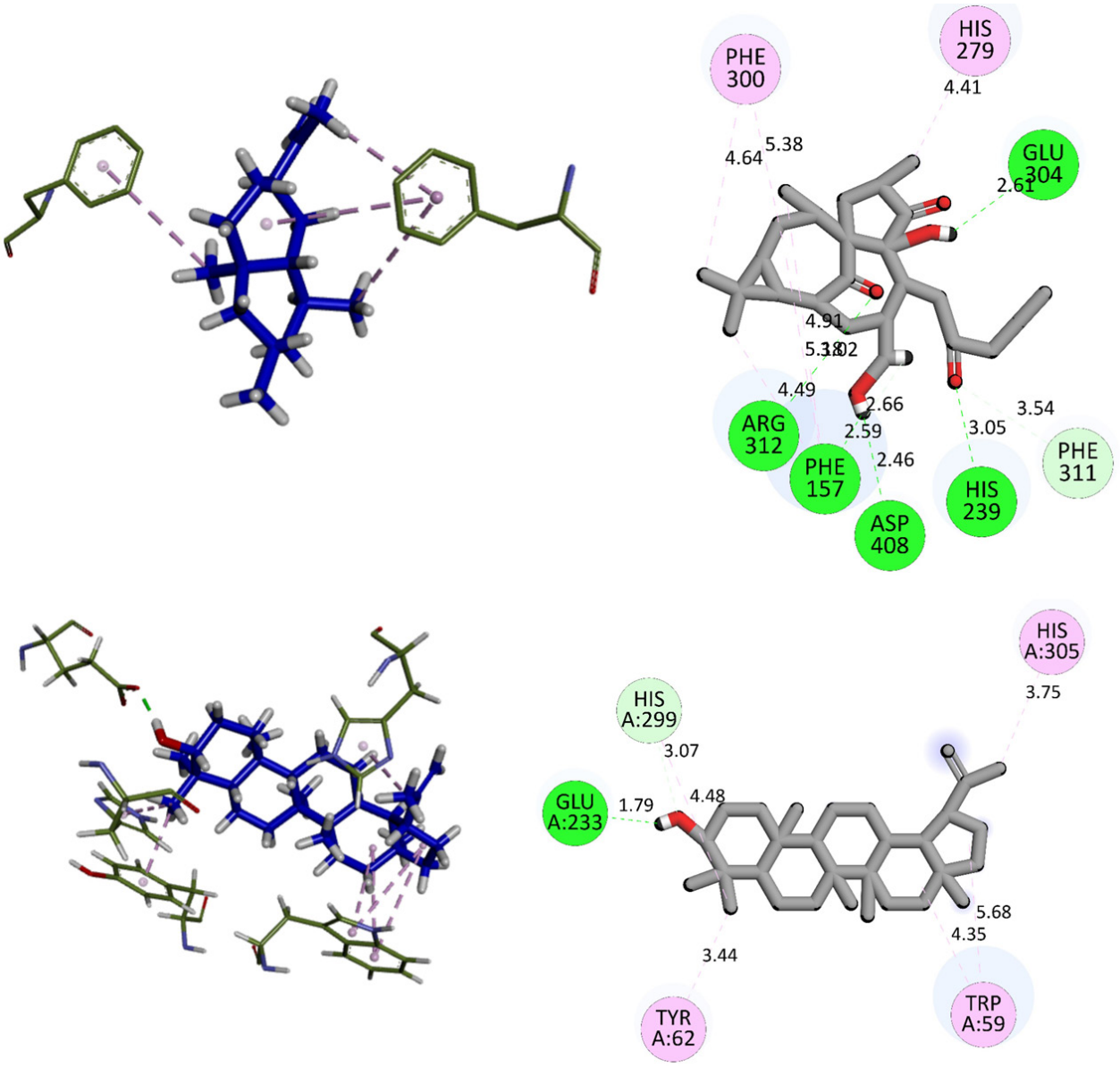

Figure 4. Results of the docking study of 2-D/3-D interaction plot of androst-5-en-17-one, 3-methoxy-,17-methoxime with α-glucosidase And interaction of lupeol with α-amylase.

interactions, and Ile A:235 and Tyr A:151 formed π-Alkyl interactions. The computed binding energy values were –6.3549, –6.9867, and –7.8347 Kcal mol.

Molecular dynamics simulations analysis

In order to find out the thermodynamic behavior of synthesized compounds on targeted proteins, molecular dynamic simulations (MDS) is one of the useful techniques to understand the stability and flexibility of ligand protein bindings. The MDS of synthesized compounds are explained in Figure 6. Through these simulation studies, it was calculated that extracted chemicals gave positive binding affinities inside the targeted proteins. α-Amyrin complex with amylase has an eigen value of 2.309341e-04. This eigen value associated with normal mode signifies the stiffness of motion. Its value correlates with the energy necessary to deform the structure. The variance value of the targeted complex indicates the increased value of variance. Covariance and elastic map also provide promising results.

Molecular docking simulations are indispensable parameters in modern drug discovery and computational biology that enable the researchers to understand molecular interactions, explore vast chemical spaces, and increase the development of new therapeutic agents.

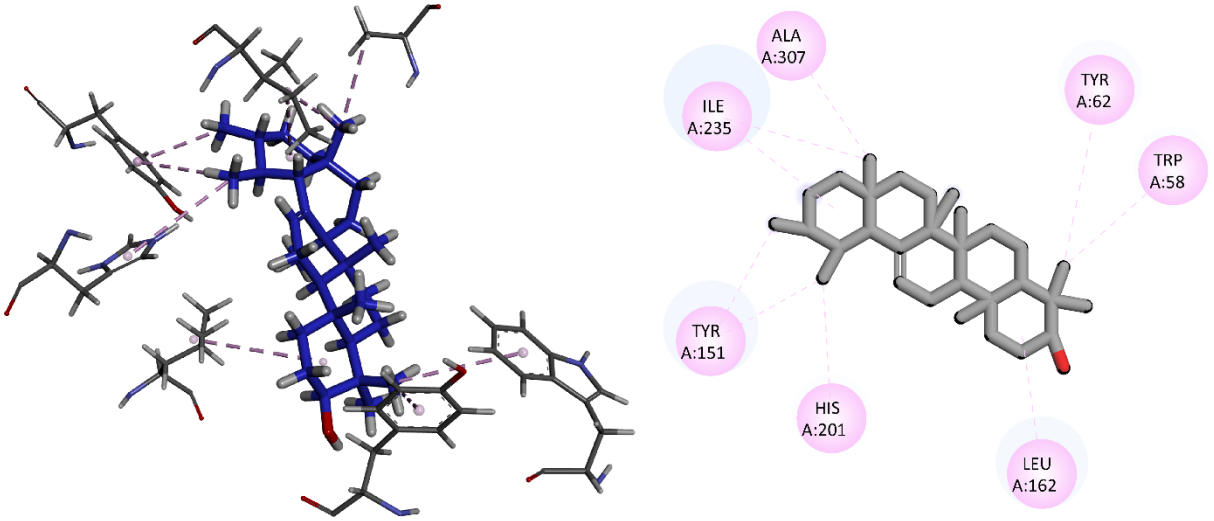

Figure 5. 2D/3D interaction of α-amyrin at the binding site of homology modeled α-amylase.

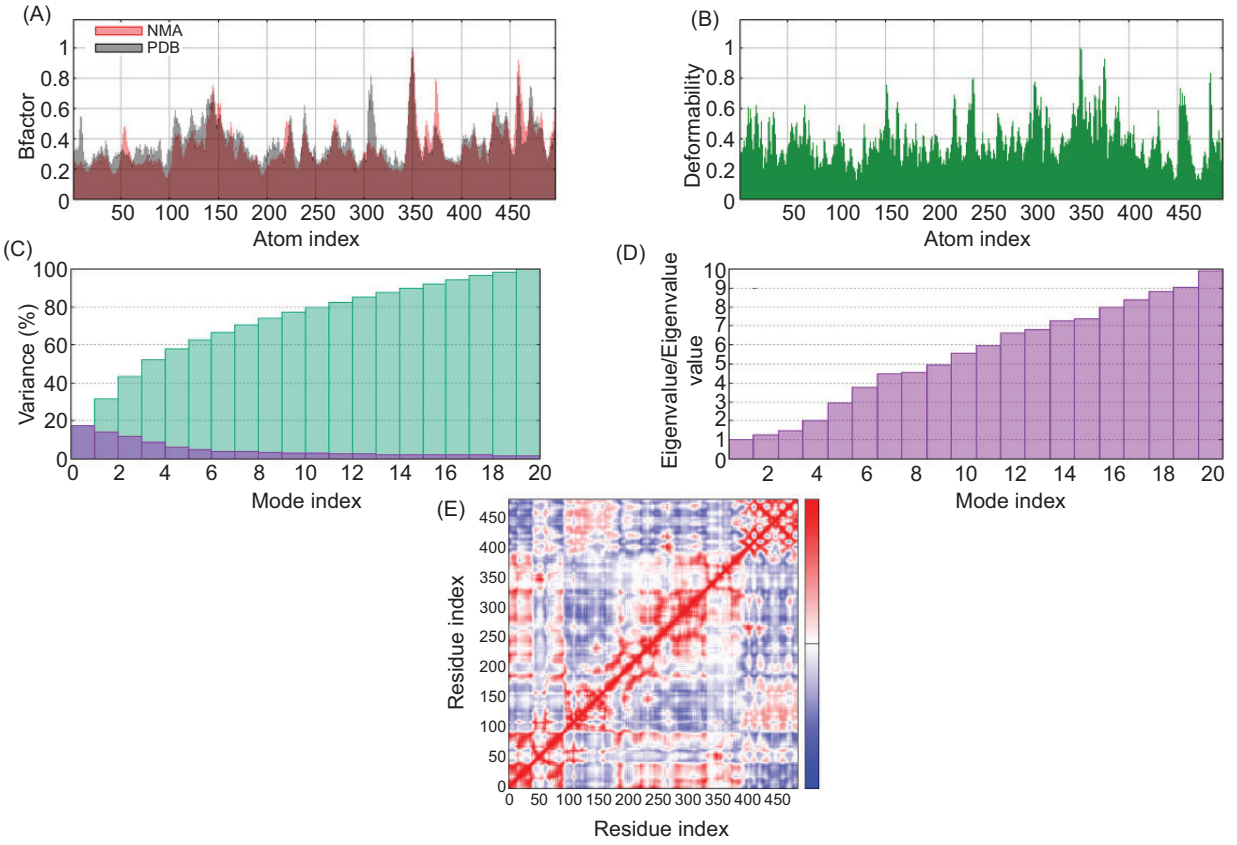

Figure 6. Molecular dynamic simulation results of α-amyrin with amylase enzyme (A-B) B-factor/mobility, (C) variance, (D) Eigen value, and (E) covariance map in which red color indicates the correlated data and white color indicates the uncorrelated data.

In vivo anti-diabetic assay

Acute toxicity test

The acute toxicity test was conducted on experimental albino mice to evaluate the toxicity profile of cones of *P. wallichiana* Pw.EtAc extract at a tested dose. No mortality or morbidity was observed and no unexpected behavioral changes or lethality was reported.

In vivo anti-hyperglycemic effect

The cones of *P. wallichiana* Pw.EtAc extracts demonstrated a hypoglycemic effect when given to diabetic mice for 28 days at dose of 150 and 300 mg/kg/day. Treating diabetic mice in the first week with the given doses of extract, a considerable glucose lowering effect was observed as compared to diabetic controls. Ethyl acetate fraction revealed the strongest hypoglycemic response at 300 mg/kg/day dose. The healthy mice were weighed and sorted into five groups, each with four animals. For the oral glucose tolerance test (OGTT) experiment, a glucose dosage of 2 g/kg body weight (bw) was given to each group. Thirty minutes after the first dose, the glucose level in the blood was measured by glucometer. After that, the sugar level was monitored for five times at every 30 min. Additionally, compared to diabetic mice not receiving medication, the administration of ethyl acetate fraction of the cones prior to OGTT lowered the rise in blood glucose level.

Following alloxan administration, diabetes in mice was confirmed by OGTT. Increase in blood glucose levels was noted in untreated diabetic group for different times, indicating the presence of diabetes. On days 7, 14, 21, and 28, the blood glucose levels were 510.33 mg/dL, 522.33 mg/dL, 515.16 mg/dL, and 522.83 mg/dL, respectively. The results obtained were compared to standard control group. Blood glucose levels decreased in mice given ethyl acetate extract at 150 mg/kg/day during the course of treatment. On day 1, the glucose level in blood was lowered to 512.16 mg/dL, 477.55 mg/dL, 359.45 mg/dL, and 254.88 mg/dL. Over the duration of 28 days, this decline persisted, reaching to 165.5 mg/dL on day 28. Blood glucose levels in mice treated with ethyl acetate fraction dose of 300 mg/kg/day also decreased significantly. The blood glucose level was 505.75 mg/dL on day 1, followed by 465.50 mg/dL on day 7, then 301.50 mg/dL on day 14, and 284.50 mg/dL and on day 21, it further decline to 131.75 mg/dL (Figure 7).

Effect of change in weight of vital organs

Variations in the weight of essential organs were observed across various groups of mice, including normal, untreated diabetic, positive control, and diabetic mice receiving both high and low doses of ethyl acetate extract. Weight of the pancreas, kidneys, heart, and liver were recorded, and the results were displayed as a mean value (average) and a standard error of mean

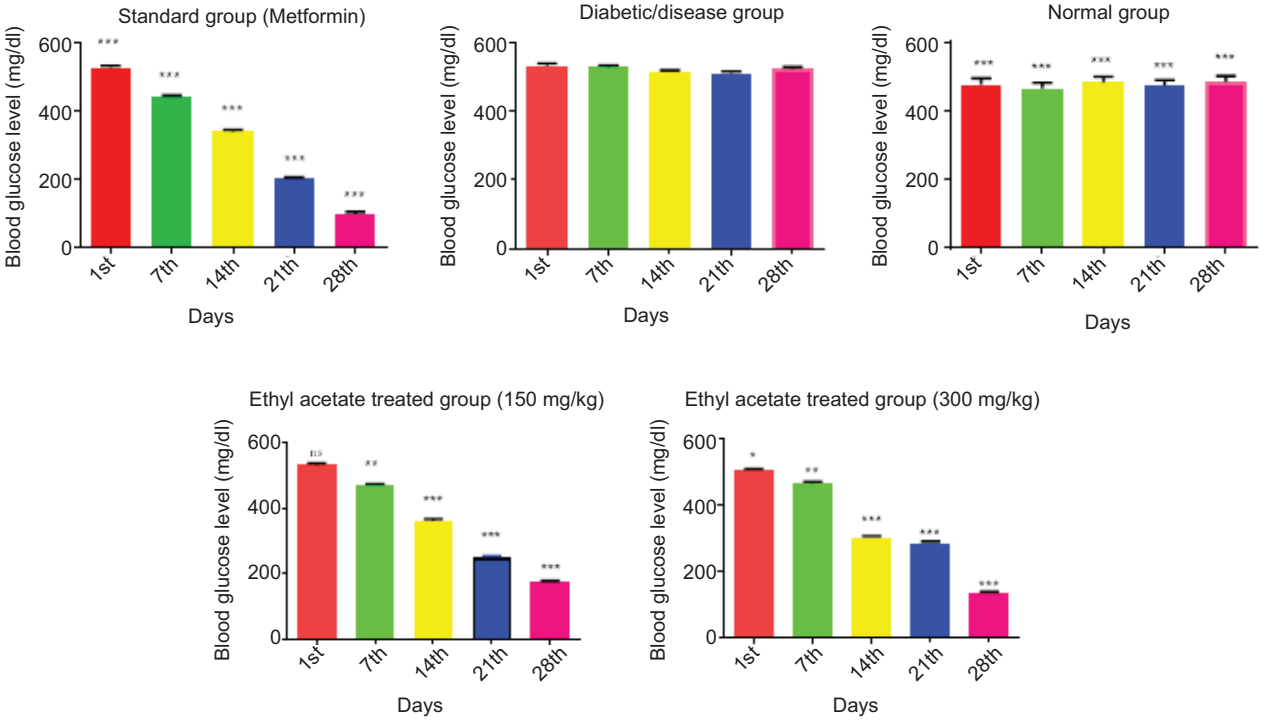

Figure 7. Result of *in vivo* antidiabetic potential of *P. wallichiana* Pw.EtAc cones extract.

as follows: 9.112±0.33 g, 0.868±0.03 g, 0.828±0.07 g, and 0.633±0.31 g, respectively. However, in the diabetic control group, the average weight was 5.727±0.20 g, 0.627±0.13 g, 0.876±0.21 g, and 0.491±0.06 g, respectively. Among diabetic mice treated with acarbose, the average weight of respective organs was 9.040±0.34 g, 1.016±0.11 g, 0.801±0.06 g, and 0.899±0.05 g. At low dose of extract of ethyl acetate, 150 mg/kg, the average weight of respective vital organ was 9.098±0.35 g, 0.708±0.09 g, 1.105±0.03 g, and 0.548±0.13 g. The diabetic group of mice treated with high dose of ethyl acetate extract, 300 mg/kg, their average weight was 0.725±0.17 g for the liver, 0.8647±0.09 g for the kidney, 1.125±0.27 g for the heart, and 0.866±0.02 g for the pancreas, as shown in Table 4.

Effect on biochemical parameters in diabetic mice

Lipid profile

The lipid profile results of diabetic group of mice treated with ethyl acetate extracts and conventional medication (acarbose) are shown in Table 5. In different groups of mice, the average cholesterol was 85.97±6.04, 97.33±3.81, 197.34±6.09, 74.25±5.05, and 68.75±2.71 mg/dL. The average triglyceride was 85.50±2.65 mg/dL in normal group, 204.25±36.65 mg/dL in diabetic group, 56.41±5.58 mg/dL in standard group, and in low and high dose groups of ethyl acetate: 91.25±5.15 mg/dL and 87.25±2.10 mg/dL, respectively. A decrease was shown in both cholesterol and triglyceride levels. In the case of HDL and LDL of normal and standard group, values were 30.75±1.57 mg/dL and 47.75±2.84 mg/dL for normal group and 28.50±2.47 and 25.75±3.35 mg/dL for acarbose treated group. The untreated diabetic group was elevated among all groups, with 42.52±4.10 mg/dL and 98.75±7.49 mg/dL. In the low and high dose of ethyl acetate extract, values were 24.75±2.03 mg/dL and 30.50±1.55 mg/dL (HDL) and 59.25±2.02 mg/dL and 41.44±1.43 mg/dL (LDL), respectively. Ethyl acetate extract had a positive impact on cholesterol levels by reducing triglycerides and LDL while increasing HDL. A high dose of extracts was found to be most effective in achieving these benefits. Diabetic peoples are at a higher risk of developing cardiovascular diseases, which can be attributed to increased levels of cholesterol in the blood. Therefore, the positive effects of the extracts on cholesterol levels may have additional benefits for diabetic patients.

Effect on renal function test

Changes in renal functions, including serum creatinine, uric acid, and blood urea are given in Table 6. Level of serum creatinine was 0.755±0.04, 0.695±0.09, 2.65±0.27, 0.67±0.08, and 0.72±0.06 mg/dL. Blood urea was higher in untreated diabetic group: 194.25±28.75 mg/dL, compared to normal group, 19.75±1.49 mg/dL. The diabetic group of animals treated with cones extract of ethyl acetate low and high dose was 25.5±2.32 mg/dL at 150 mg/kg. The decrease was observed in blood urea of treated group at a dose of 300 mg/kg (21.5±1.55 mg/dL). Uric acid was not increased in any of the treated and untreated groups.

Effect on Liver function test

The results of ethyl acetate and acarbose treatment on liver enzymes, including serum bilirubin, SGPT, ALT, and ALP, among different groups of mice are summarized in Table 7. In the case of diabetic control, the serum bilirubin was 1.695±0.42 mg/dL, 0.68±0.914 mg/dL for normal control, 0.625±0.06 mg/dL for acarbose standard drug, for extract fraction at 150 mg/kg, 0.75 ± 0.04 mg/dL, and at 300 mg/kg, 0.67 ± 0.08 mg/dL. Among liver function enzymes,

Table 4. Effect of ethyl acetate extract treatment on the weight (g) of vital organs of mice.

Vital organs	Acarbose, 5 mg/kg	Normal control	Diabetic control	Pw.EtAc 150 mg/kg	Pw.EtAc 300 mg/kg
Liver	9.040±0.34	9.112±0.33	5.727±0.20	9.098±0.35	0.725±0.17
Kidney	1.016±0.11	0.868±0.03	0.627±0.13	0.708±0.09	0.864±0.09
Heart	0.801±0.06	0.828±0.07	0.876±0.21	1.105±0.03	1.125±0.27
Pancreas	0.889±0.05	0.633±0.31	0.491±0.06	0.548±0.13	0.866±0.02

Table 5. Effect of treatment on the lipid profile of different groups of diabetic animals.

Lipid profile	Acarbose (5 mg/kg)	Normal control	Diabetic control	Pw.EtAc 150 mg/kg	Pw.EtAc 300 mg/kg
S. cholesterol	97.33±3.81	85.97±6.04	197.34±6.09	74.25±5.05	68.75±2.71
Triglyceride	56.41±5.58	85.50±2.65	204.25±6.65	91.25±5.15	87.25±2.10
HDL	28.50±2.47	30.75±1.57	42.52±4.10	24.75±2.03	30.50±1.55
LDL	25.75±3.35	47.75±2.84	98.75±7.49	59.25±2.02	41.44±1.43

Table 6. Effect of treatment on renal function biomarker.

Renal function test	Acarbose (5 mg/kg)	Normal control	Diabetic control	Pw.EtAc 150 mg/kg	Pw.EtAc 300 mg/kg
S. creatinine	0.775±0.04	0.695±0.09	2.65±0.27	0.76±0.08	0.72±0.06
Blood urea	19.75±1.49	20.75±0.10	194.25±28.74	25.5±2.32	21.5±1.55
Uric acid	2.05±0.330	2.58±0.182	2.95±0.326	2.78±0.424	2.52±0.143

Table 7. Effect of cones treatment on liver function biomarkers.

Liver function test	Normal control	Diabetic control	Acarbose (5 mg/kg)	Pw.EtAc 150 mg/kg	Pw.EtAc 300 mg/kg
Serum bilirubin (mg/dL)	0.68± 0.914	1.695±0.42	0.625±0.06	0.75±0.04	0.67±0.08
SGPT (UL^{-1})	22.5±2.53	59±6.12	26.25±2.65	21.5±3.88	24.25± 2.28
Alkaline phosphatase (ALP, UL^{-1})	128±6.41	228.25±43.39	112.75±1.88	119±14.05	124±8.45
Aspartate transaminase (AST, UL^{-1})	37.25±2.03	63.25±3.16	47.75±2.39	52.75± 1.79	47.25±1.79

Note: Values were mean and SEM.

SGPT was 22.5±2.53 UL^{-1} (normal control), 59±6.12 UL^{-1} (diabetic control), 26.25±2.65 UL^{-1} (acarbose), and 21.5±3.88 UL^{-1} and 24.25±2.28 UL^{-1} for plant extract's low and high doses. The average concentration of ALP was 128±6.41 UL^{-1} in normal control, 228.25±43.39 UL^{-1} in diabetic control, 112.75±1.88 UL^{-1} in acarbose treated group, 119±14.05 UL^{-1} and 124±8.45 UL^{-1} in low and high dose treated extract.

Histopathology

Effect on histology of pancreas and heart

In control group, the pancreas showed normal acini and cellular population of the Islets of Langerhans. The diabetic group showed irregular and damaged Islets of Langerhans, as shown in Figure 8. The acarbose-treated group showed moderate increase in the size of islet cells and cellular population. No recovery of cellular organization and size of the Islets of Langerhans was observed, and there was no significant regenerative effect on the damaged pancreatic tissue in the group treated with a low dose. However, with a higher dose of *P. wallichiana* Pw.EtAc fraction cones (300 mg/kg/day), there was a partial restoration of cellular population and size of the Islets of Langerhans, which had a greater impact on the rejuvenation of β cells, compared to the lower dose.

The normal group of albino mice showed normal histology of the heart. The diseased diabetic group revealed severe injuries to the myocardium, and cellular infiltration, signifying inflammation in the heart tissue. In comparison to the diabetic group, the standard group's cellular infiltration severity was significantly reduced. At low dose, cones extract showed no significant reduction in cellular infiltration, and myocarditis was observed. No modification was found in the heart tissue, revealing that a higher dose did not negatively impact heart histology, as shown in Figure 9.

Effect on histology of liver and kidney

The liver exhibited normal hepatocytes and liver architecture in normal control group. Albino mice belonging to the diabetic group exhibited an increase in vacuolation within the cytoplasm of hepatocytes. These vacuoles were demarcated as vague, transparent vacuoles, which are usually a sign of diabetes-related glycogen invasion, as shown in Figure 10. After treatment with acarbose, hepatocytes and normal hepatic architecture showed normal restoration with few minor vacuolation of hepatocytes. The standard treatment had a positive effect with some persisted glycogen infiltration. The group treated with 150 mg/kg showed hepatocytes restoration, dilated central vein but did not prevent glycogen infiltration. In another group treated with high dose showed a slightly dilated central vein with normal hepatic architecture and hepatocytes with slight glycogen infiltration.

In the control group, displayed regular renal tubules and glomerulus looked normal. Mice in the diabetic group showed kidney damage linked with diabetes, that is, loss of renal tubules and swelling in Bowman's space due to glomerular damage. The acarbose-treated group showed restoration in glomerulus and renal tubules. The group treated with a lower dose (150 mg/kg/day) exhibited normal glomeruli with tubular inflammation, demonstrating some beneficial effects in stabilizing the glomerular structure; however, it did not entirely avert inflammation in the renal tubules. In contrast, the higher dose group displayed a more pronounced regenerative impact on

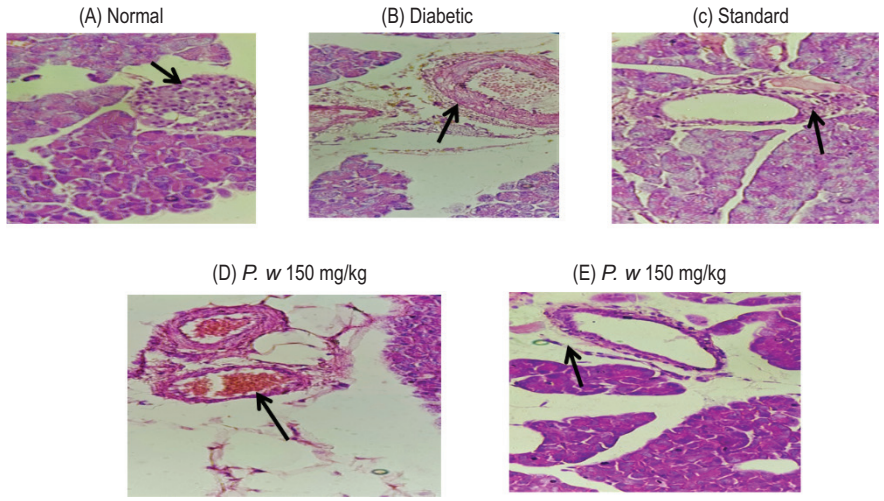

Figure 8. Histopathology of the pancreas in treatment with ethyl acetate fraction cones extract in different groups of diabetic mice.

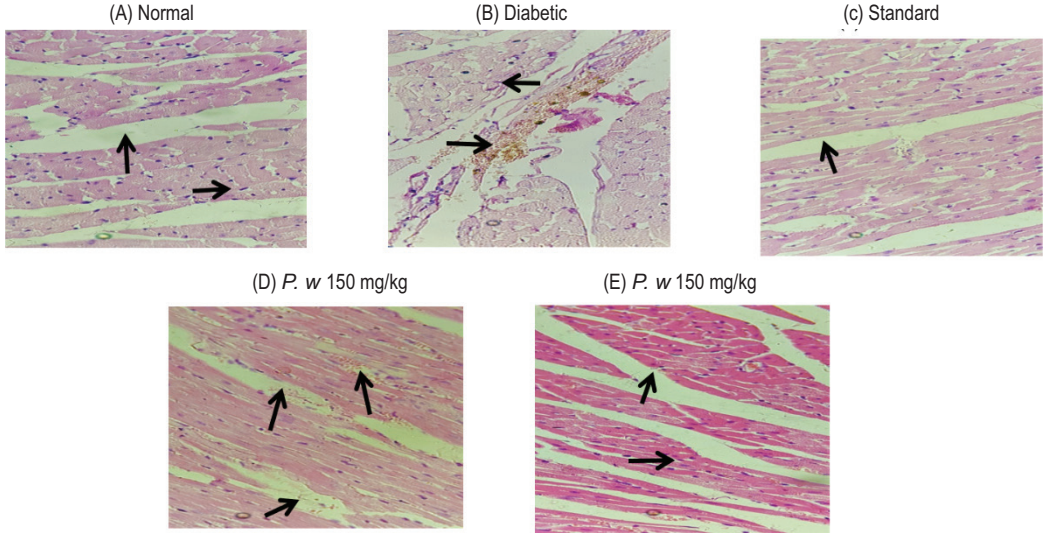

Figure 9. Treatment of ethyl acetate extract in histology of the heart.

kidney's histopathology, effectively restoring the normal structure of renal tubules and glomeruli as evidenced by the findings in Figure 11.

Discussion

Hyperglycemia, or elevated blood glucose levels, is a characteristic of diabetes. Prolonged hyperglycemia can lead to various complications. Additional 96,000 cases of TIDM are diagnosed annually in children and adolescents worldwide, according to International Diabetes Federation (IDF), with the highest numbers reported in the countries such as the United State, China, India, Russia, Nigeria, Brazil, the United Kingdom, Saudi Arabia, Germany, and Algeria; these countries collectively account for 60% new cases (Zheng, Luan *et al.,* 2020). In 2017, an estimated 425 million adults, aged 20–79 years, were living with diabetes globally. This indicates a widespread prevalence of the disease across various age groups. Certain ethnic groups, particularly those with higher rates of obesity, have a greater prevalence of diabetes (Schulze and Hu 2022). This emphasizes the complex interplay between genetic predisposition, lifestyle factors, and socioeconomic determinants of health.

GC-MS is a diagnostic method used to categorize and measure the biochemical compounds present in a cones

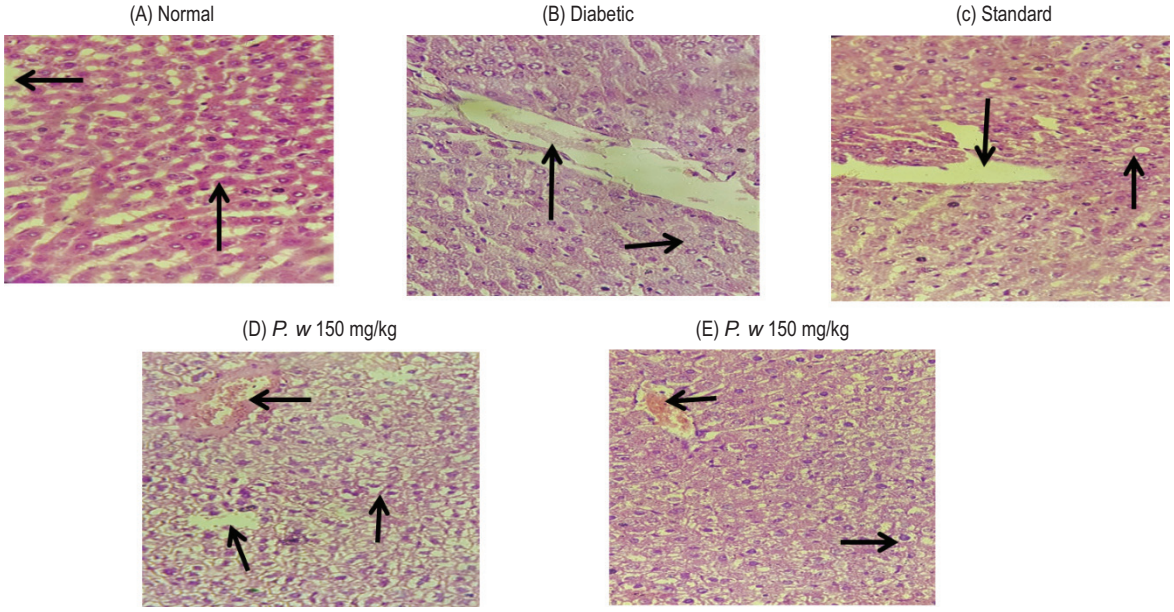

Figure 10. Histopathology of the liver in treatment of cones extract in different groups of diabetic mice.

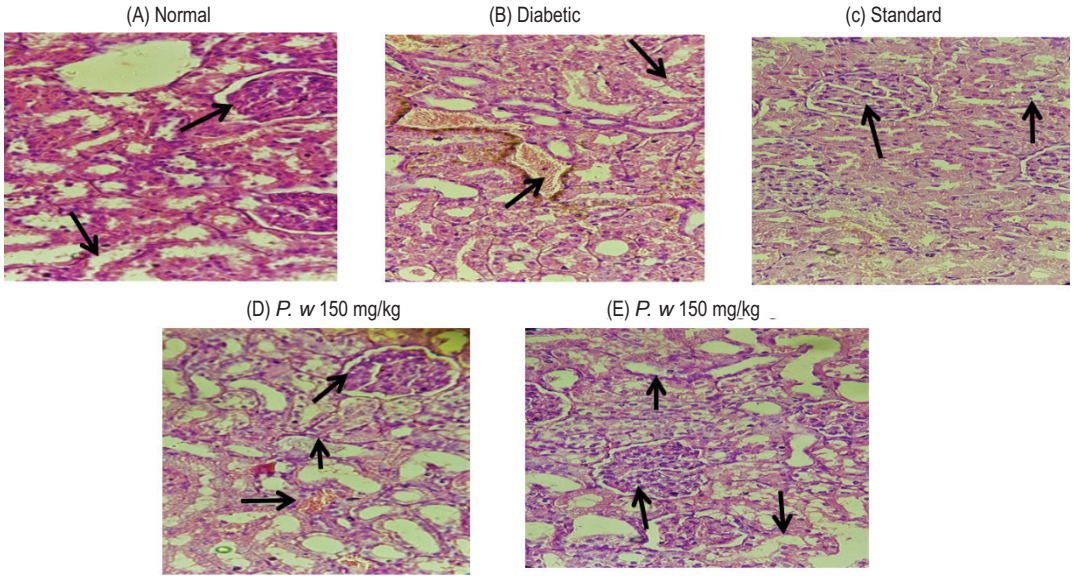

Figure 11. Histopathology of the kidney.

sample. The extracts from *P. wallichiana* contain 45 compounds, and a variety of phytochemicals, including phenols and flavonoids. These compounds are known for their potential health benefits. The occurrence of these various compound classes in extracts may possess therapeutic applications. These may include antimicrobial, antioxidant, anticancer, anti-inflammatory, and antidiabetic potential. Pregnane-3,11,17,20,21-pentol, cyclic 17,21-(methylboronate), (3à,5á,20R) reported it may have a varied range of pharmacological effects. Pregnane is a class of natural or synthetic organic compounds that are often associated with steroid hormones. This compound may have antidiabetic properties that affect blood sugar levels and insulin regulation. Obesity-related issues are also influenced by compounds that affect metabolism and appetite regulation (Abdel-Sattar and Ali 2022). α-Amyrin, a triterpenoid compound, originates naturally in many plant sources, including medicinal herbs. It has been studied for its ability to help regulate blood sugar levels. It may have antihyperglycemic and hypolipidemic effects, which mean, it can lower elevated blood sugar levels and lower lipid (fat) levels in the blood. High levels of lipids, including cholesterol and triglycerides, are linked with a risk of cardiovascular diseases. By reducing

lipid levels, α-amyrin can contribute to improve lipid metabolism and the overall cardiovascular health (Achi and Ohaeri 2015).

Antioxidants are linked to a decreased risk of serious diseases, such as cancer, diabetes, and cardiovascular diseases. They also lead to the antioxidant defense system that protects cells from oxidative stress-related damage. Naeem *et al.* (2010) stated that *P. wallichiana* bark's methanolic fraction contains flavanols in substantial quantities. Flavanols are a type of flavonoid known for their antioxidant properties. Maimoona *et al.* (2011) found *P. wallichiana* phytochemicals and antioxidant in different extracts. Dar *et al.* (2012) investigated the essential oil isolated from *P. wallichiana* in Kashmir and found it to have durable anti-proliferative potential in contradiction of five human cancer cell lines. It also exhibited radical scavenging activity and attributed to the redox properties of phenolic and flavonoid compounds, which act as reducing, scavenging, and chelating agents.

A comparison of obtained results was done with other reported results, particularly total phenolic and flavonoid content, antioxidant assays (DPPH and ABTS), and antidiabetic agents targetting α-amylase and α-glucosidase. The cones of *P. wallichiana* extracts showed varying TPC and TFC values with different solvents. In the reported research, TPC values were found to be 4.09 mg GAE/g for ethanolic extract and 4.06 mg GAE/g for n-butanol extract. In comparison, our phytochemical studies showed higher TPC and TFC values (28.55 mg GAE/g and 63.05 mg QE/g, respectively) for ethyl acetate extract (Naeem, Taskeen *et al.,* 2010).

Among bark and leaf extracts of methanol and aqueous fractions of *P. wallichiana*, maximum antioxidant activity was monitored with IC_{50} of 20.83 μg/mL and 25.9 μg/mL respectively. A study described the combination of essential oils from needles and stems, showing potent antioxidant activity (47.19%) at a concentration of 100 μg/mL, and this activity was compared to the standard ascorbic acid, exhibiting a high level of antioxidant activity, which was 78.7% in one study conducted by (Qadir and Shah 2014). Additionally, in our study, all fractions of *P. wallichiana* cones demonstrated ability to scavenge free radicals, indicating their potential of antioxidant activity. The ethyl acetate extract showed particularly low IC_{50} values (13 μg/mL [DPPH] and 5.3 μg/mL [ABTS assay]). Ethyl acetate fraction exhibited the highest percentage inhibition of α-amylase and α-glucosidase, that is 79.55±0.49 μg/mL and 88.56±0.07 μg/mL.

Alloxan specifically targets beta cells of the pancreas, which produces insulin. The administration of alloxan leads to oxidative stress, beta-cell death, and a decrease in insulin production, resulting in insulin-dependent diabetes (Sabir, Saleem *et al.,* 2018). Many scientific papers have reported that hyperlipidemia coexisted with hyperglycemia in diabetics (Choi, Kim *et al.,* 2002). The diabetic albino mice's blood glucose levels decreased significantly after treatment with ethyl acetate extracts. This reduction was statistically significant if related to the diabetic control group and was known to the extract's hypoglycemic constituents. The lipid metabolism was positively impacted by ethyl acetate extracts. In diabetic mice, ethyl acetate extracts increased HDL levels and decreased LDL, triglycerides, and cholesterol (Schulze and Hu 2022). Triglycerides and cholesterol dropped significantly with administration of ethyl acetate extract, indicating the possibility of hypoglycemic and hypolipidemic conditions. The extract's capacity to restore metabolic state and its antidiabetic qualities probably improved these parameters.

Liver marker enzymes were used to determine LFT. Only liver, muscles, kidney, placenta, and bones contain AST and ALP (Burroughs and Westaby 2005). As an indication of hepatic damage, AST is implicated in the catalysis of alanine to pyruvate and glutamate. When cells are injured, the cytoplasm containing enzymes called aminotransferases and alkaline phosphatases are released. They act as an indicator of the loss of integrity of the cell membrane (Moss 1975). In diabetic mice, alloxan cause an increase in the level of ALP and AST enzymes, whereas, the extract and Acarbose treated animals hav elowere ALP and AST levels. Hepatic injury was observed due to the increased concentration of these enzymes in the liver. The extract's capacity to lower effectively ALP points to its hepatoprotective properties and indicates an early improvement of the liver's secretary phenomenon. The extract's hepatoprotective properties were due to the presence of flavonoid's capacity to scavenge free radicals.

Serum ALP and AST levels rise in diabetic mice induced by alloxan, indicating hepatic damage. This is due to liver's high concentration of these enzymes. Restoration of AST and ALP levels following treatment with ethyl acetate extract and acarbose indicated recovery from liver injury. The extract's hepatoprotective properties could be attributed to the existence of flavonoids, presumably because of their capacity to scavenge free radicals. Increased blood urea and creatinine levels were symptomatic of renal failure in diabetic mice. Elevated urea levels were probably due to enhanced protein catabolism or amino acid deamination for gluconeogenic synthesis (Arkkila, Koskinen *et al.,* 2001). Renal function was improved by a prolonged exposure of extract, which caused a considerable decrease in serum urea and creatinine levels. The extract's potential to prevent diabetes and the renal tubule's capacity to regenerate themselves could be responsible for this improvement (Yamaguchi and Weitzmann 2009).

Conclusions

We can assert with scientific evidence that we have investigated the cones of *Pinus wallichiana* for the first time in relation to diabetes management, particularly concerning the protection of vital organs. Moreover, the results of free radical scavenging were sufficiently promising to supplement antidiabetic findings by safeguarding the beta cells of pancreas, which are the production center of insulin. It can be stated that the cones of *P. wallichiana* are rich in various phytocomponents and possess potential antidiabetic properties.

Acknowledgments

The authors extend their appreciation to the Deanship of Research and Graduate Studies at King Khalid University for funding this work through Large Research Project under Grant No. RGP2/258/46.

Data Availability

The whole data related to *P. wallichiana* cones of which this manuscript is a part can be found in the PhD thesis of Ms. Nigar Aksar submitted to the Higher Education Commission of Pakistan.

Mandatory Disclosure on Use of Artificial Intelligence

The authors declare that no AI-assisted tools were used in the preparation of this manuscript. All references have been manually verified for accuracy and relevance.

Author Contributions

Nigar Aksar, Muhammad Fiaz Khan, and Shumaila Noreen contributed to plant collection, phytochemical studies, and sample preparations. Nigar Aksar performed *in vitro* assays with contributions of Muhammad Fiaz Khan, Mushtaq Ahmad Mir, Nasreena Bashir, Abdul Sadiq, Muhammad Ayaz, and Farhat Ullah. Nigar Aksar performed the *in vivo* and molecular docking studies, manuscript drafting, and revision. All the authors had read and approved the final version of the paper for publication.

Conflict of Interest

The authors declared that they had no conflict of interest.

References

Abdel-Sattar, E. and Ali, D.E. 2022. "Russelioside B: a pregnane glycoside with pharmacological potential." Rev Bras Farmacogn. 32(2):188–200. https://doi.org/10.1007/s43450-022-00245-x

Achi, N.K. and Ohaeri, O. 2015. GC-MS determination of bioactive constituents of the methanolic fractions of *Cnidoscolus aconitifolius*. Br J Pharm Res. 5(3):163. https://doi.org/10.9734/BJPR/2015/13893

Ahuja, J., Suresh, J. and Deep, A. 2011. Phytochemical screening of aerial parts of *Artemisia parviflora*. Der Pharm Lett. 3(6):116–124.

Akinjogunla, O., Yah, C., Eghafona, N. and Ogbemudia, F. 2010. Antibacterial activity of leave extracts of Nymphaea lotus (Nymphaeaceae) on methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) isolated from clinical samples. Ann Biol Res. 1(2):174–184.

Arkkila, P.E., Koskinen, P.J., Kantola, I.M., Rönnemaa, T., Seppänen, E. and Viikari, J.S. 2001. Diabetic complications are associated with liver enzyme activities in people with type 1 diabetes. Diabetes Res Clin Pract. 52(2):113–118. https://doi.org/10.1016/S0168-8227(00)00241-2

BP, C., Chaware, V., Joshi, Y. and Biyani, K. 2009. Hepatoprotective activity of Hydroalcoholic extract of *Momordica charantia* Linn. leaves against carbon tetrachloride induced hepatopathy in rats. Inter. J ChemTech Res. 1(2):355–358.

Burroughs, A. and Westaby, D. 2005. Liver, biliary tract and pancreatic disease. In: Kumar, P. and Clarke (Eds.) Clinical Medicine, 6th Edn. pp. 347–418.

Chaturvedi, O. and Pandey, N. 2001. Genetic divergence in *Bombax ceiba* L. germplasms. Silvae Genet. 50(3–4):99–102.

Choi, C.W., Kim, S.C., Hwang, S.S., Choi, B.K., Ahn, H.J., Lee, M.Y., Park, S.H. and Kim, S.K. 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci. 163(6): 1161–1168. https://doi.org/10.1016/S0168-9452(02)00332-1

Dallakyan, S. and Olson, A.J. 2015. Small-molecule library screening by docking with PyRx. In *Chemical biology: methods and protocols*, pp. 243–250. New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-2269-7_19

Dar, M. Y., W. A. Shah, S. Mubashir and M. A. Rather 2012. Chromatographic analysis, anti-proliferative and radical scavenging activity of Pinus wallichina essential oil growing in high altitude areas of Kashmir, India. Phytomedicine 19(13): 1228–1233. https://doi.org/10.1016/j.phymed.2012.07.015

Edelman, S.V. 1998. Type II diabetes mellitus. Adv Intern Med. 43:449–500.

Eisenbarth, G.S. 1986. Type I diabetes mellitus. New Engl J Med. 314(21):1360–1368. https://doi.org/10.1056/NEJM198605223142106

Guex, N. and Peitsch, M.C. 1997. SWISS-MODEL and the Swiss-Pdb viewer: an environment for comparative protein modeling. Electrophoresis. 18(15):2714–2723. https://doi.org/10.1002/elps.1150181505

Hanwell, M.D., Curtis, D.E., Lonie, D.C., Vandermeersch, T., Zurek, E. and Hutchison, G.R. 2012. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. J Cheminform. 4(1):1–17. https://doi.org/10.1186/1758-2946-4-17

Hussain, F., Khan, Z., Jan, M.S., Ahmad, S., Ahmad, A., Rashid, U., Ullah, F., Ayaz, M. and Sadiq, A. 2019. Synthesis, *in vitro*. α-glucosidase inhibition, antioxidant, *in vivo* antidiabetic and molecular docking studies of pyrrolidine-2, 5-dione and thiazolidine-2, 4-dione derivatives. Bioorg Chem. 91:103128. https://doi.org/10.1016/j.bioorg.2019.103128

Kaul, K., Tarr, J.M., Ahmad, S.I., Kohner, E.M. and Chibber, R. 2013. Introduction to diabetes mellitus. Diabetes: an old disease, a new insight: pp. 1–11. https://doi.org/10.1007/978-1-4614-5441-0_1

Kodangala, C., Saha, S. and Kodangala, P. 2010. Phytochemical studies of aerial parts of the plant *Leucas lavandulaefolia*. Der Pharma Chemica. 2(5):434–437.

Kumar, N. 2015. Antioxidant and antibacterial properties of leaves of *Elaeocarpus sphaericus* Roxb. and *Pinus wallichiana* from Uttarakhand region of India. Int J Green Pharm (IJGP). 9(4). https://doi.org/10.22377/ijgp.v9i4.570

Linga Rao, M. and Savithramma, N. 2011. Phytochemical studies of *Svensonia hyderobadensis* (Walp.) mold: a rare medicinal plant. Der Pharm. Lett. 3:51–55.

Little, E.L. and Critchfield, W.B. 1969. Subdivisions of the Genus *Pinus* (Pines). Monograph. US Department of Agriculture, Forest Services, Washington, DC.

Mahnashi, M.H., Alqahtani, Y.S., Alqarni, A.O., Alyami, B.A., Jan, M.S., Ayaz, M., Ullah, F., Rashid, U. and Sadiq, A. 2021. Crude extract and isolated bioactive compounds from *Notholirion thomsonianum* (Royale) Stapf as multitargets antidiabetic agents: *in vitro* and molecular docking approaches. BMC Compl Med Ther. 21:1–13. https://doi.org/10.1186/s12906-021-03443-7

Maimoona, A., Naeem, I., Saddiqe, Z., Ali, N., Ahmed, G. and Shah, I. 2011. Analysis of total flavonoids and phenolics in different fractions of bark and needle extracts of *Pinus roxburghii* and *Pinus wallichiana*. J Med Plants Res. 5(13):2724–2728.

Malviya, N., Jain, S. and Malviya, S. 2010. Antidiabetic potential of medicinal plants. Acta Pol Pharm. 67(2):113–118.

Moss, D. 1975. Alkaline phosphatase isoenzymes. Tech Clin Aspects Enzyme. 20(1):20–34. https://doi.org/10.1159/000458916

Naeem, I., Taskeen, A., Mubeen, H. and Maimoona, A. 2010. Characterization of flavonols present in barks and needles of *Pinus wallichiana* and *Pinus roxburghii*. Asian J Chem. 22(1):41.

Nawaz, A., Sadiq, A., Bashir, N., Rashid, U., Ullah, F., Khan, S., Ullah, F., Khan, M.I. and Ayaz, M. 2025. Synthetic derivates of progesterone ameliorate scopolamine-induced cognitive deficits in animal models: antioxidant, enzyme inhibitory, molecular docking and behavioral correlates. Curr Neuropharmacol. 23(1):1797–1812. https://doi.org/10.2174/011570159X357722250212094900

Noor, A., Gunasekaran, S., Manickam, A.S. and Vijayalakshmi, M.A. 2008. Antidiabetic activity of Aloe vera and histology of organs in streptozotocin-induced diabetic rats. Curr Sci. 94(8):1070–1076.

Olokoba, A.B., Obateru, O.A. and Olokoba, L.B. 2012. Type 2 diabetes mellitus: a review of current trends. Oman Med J. 27(4):269. https://doi.org/10.5001/omj.2012.68

Pradeep, A., Dinesh, M., Govindaraj, A., Vinothkumar, D. and Ramesh Babu, N. 2014. Phytochemical analysis of some important medicinal plants. Int J Biol Pharm Res. 5(1):48–50.

Qadir, M. and Shah, W.A. 2014. Comparative GC-MS analysis, antioxidant, antibacterial and anticancer activity of essential oil of Pinus wallichaina from Kashmir, India. Elixir Appl Chem. 72:25819–25823.

Rahman, T.U., Uddin, G., Khattak, K.F., Liaqat, W. and Choudhary, M.I. 2016. Antibacterial, antifungal, insecticidal and phytotoxic activities of leaves of *Pinus wallichiana*. J Chem Pharm Res. 8(1):420–424.

Sabir, S., Akhtar, M.F. and Saleem, A. 2019. Endocrine disruption as an adverse effect of non-endocrine targeting pharmaceuticals. Environ Sci Pollut Res. 26:1277–1286. https://doi.org/10.1007/s11356-018-3774-4

Sabir, S., Saleem, A., Akhtar, M.F., Saleem, M. and Raza, M. 2018. Increasing beta cell mass to treat diabetes mellitus. Adv Clin Exp Med. 27(9):1309–1315. https://doi.org/10.17219/acem/74452

Sadiq, A., Zeb, A., Ullah, F., Ahmad, S., Ayaz, M., Rashid, U. and Muhammad, N. 2018. Chemical characterization, analgesic, antioxidant, and anticholinesterase potentials of essential oils from Isodon rugosus Wall. ex. Benth. Front Pharmacol. 9:623. https://doi.org/10.3389/fphar.2018.00623

Safhi, M.M., Alam, M.F., Sivakumar, S.M. and Anwer, T. 2019. Hepatoprotective potential of *Sargassum muticum* against STZ-induced diabetic liver damage in wistar rats by inhibiting cytokines and the apoptosis pathway. Anal. Cell Pathol. 1:7958701 https://doi.org/10.1155/2019/7958701

Schulze, M.B. and Hu, F.B. 2022. Epidemiology of diabetes. In: Handbook of Epidemiology. Springer, New York, NY: NY: Springer, pp. 1–49. https://doi.org/10.1007/978-1-4614-6625-3_66-1

Singh, L., Dixit, P., Srivastava, R.P., Pandey, S., Verma, P.C. and Saxena, G. 2019. Ethnobotany and pharmacology of Pinus species growing naturally in Indian Himalayas: a plant review. Curr Pharm Biotechnol. 20(15):1281–1287. https://doi.org/10.2174/1389201020666190819153600

Vinci, G., D'Ascenzo, F., Maddaloni, L., Prencipe, S.A. and Tiradritti, M. 2022. The influence of green and black tea infusion parameters on total polyphenol content and antioxidant activity by ABTS and DPPH assays. Beverages. 8(2):18. https://doi.org/10.3390/beverages8020018

Wan Mohd Zain, W.Z., Ramli, N.N., Jusoh, S. and Hamid, N.A. 2021. Antioxidant activity, total phenolic and flavonoid content from leaves and seed extracts of *Hevea brasiliensis* clone. J Academia. 9:1–7.

Yamaguchi, M. and Weitzmann, M.N. 2009. The bone anabolic carotenoid β-cryptoxanthin enhances transforming growth factor-β1-induced SMAD activation in MC3T3 preosteoblasts. Int J Mol Med. 24(5):671–675. https://doi.org/10.3892/ijmm_00000278

Zheng, J.-S., Luan, J.A., Sofianopoulou, E., Sharp, S.J., Day, F.R., Imamura, F., Gundersen, T.E., Lotta, L.A., Sluijs, I and Stewart, I.D. 2020. The association between circulating 25-hydroxyvitamin D metabolites and type 2 diabetes in European populations: a meta-analysis and Mendelian randomisation analysis. PLoS Med. 17(10):e1003394. https://doi.org/10.1371/journal.pmed.1003394

Zulfqar, F., Akhtar, M.F., Saleem, A., Akhtar, B., Sharif, A. and Saleem, U. 2020. Chemical characterization, antioxidant evaluation, and antidiabetic potential of *Pinus gerardiana* (Pine nuts) extracts. J Food Biochem. 44(6):e13199. https://doi.org/10.1111/jfbc.13199