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Potentilla fulgens Wall ex Sims. exerts anti-diabetic effects by inhibiting α-amylase and α-glucosidase: deeper insights through molecular docking

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ABSTRACT

Potentilla fulgens Wall ex Sims., a local medicinal plant used by the Khasi tribe of Meghalaya, India, has been reported to be rich in tannins, polyphenols, triterpenoids, and flavonoids. Although several studies have been conducted on its antidiabetic and anti-oxidant properties, most reports were done with crude polar extracts. In this study, we report the inhibitory effect of the non-polar chloroform extract of *P. fulgens* (NPFE) on α- amylase and α- glucosidase. The extract exhibited a potent antioxidant effect comparable to the reference standard as reflected by the IC_{50} values in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Further, the antihyperglycemic action of NPFE was observed in alloxan-induced diabetic mice from the Intraperitoneal Glucose Tolerance Test (IPGTT). Spectral and chromatographic analysis using FTIR and GC-MS/MS showed the presence of important functional groups and bioactive compounds. In silico molecular docking of the identified bioactive compounds carried out against α-amylase and α-glucosidase provided more insights into its antihyperglycemic properties.

1. Introduction

Diabetes, a chronic metabolic disorder is characterized by high glucose levels in the blood, which occur when the body either cannot produce enough insulin or cannot use it effectively (Powers & D'Alessio, 2018). As per the IDF Atlas published in 2021, more than 537 million adults worldwide are living with diabetes, which constitutes about 10.5% of the global adult population. The report highlights the alarming rise in diabetes prevalence globally. Projections indicate that by 2045, the number of adults with diabetes could reach 783 million, or approximately 12.2 % of the global population. People with diabetes face higher risks of various health complications, including heart disease, peripheral arterial disease, stroke, obesity, cataracts, erectile dysfunction, and non-alcoholic fatty liver disease (Kazi & Blonde, 2001).

Carbohydrates play a crucial role as a primary energy source for survival, and their digestion begins in the mouth and continues in the intestines (Jequier, 1994). These carbohydrates are

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broken down into absorbable monomers through the action of enzymes such as α-amylase and α-glucosidase. This digestive process can lead to postprandial hyperglycemia (Björck et al., 1994), which, if unmanaged, can contribute to the development of diabetes (Ceriello, 2005; van Dijk et al., 2011).

Carbohydrate digestibility is closely linked to increased postprandial blood glucose levels. One effective approach to mitigating postprandial hyperglycemia is to inhibit carbohydrate-digesting enzymes in the intestinal tract. α-Amylase present in the saliva and pancreas is a critical enzyme that breaks down carbohydrates by hydrolyzing α-1,4-glucan linkages in starch, maltodextrins, and other similar polysaccharides, resulting in shorter oligomers (Truscheit et al., 1981). Additionally, α-glucosidase, found in the human intestinal mucosal cells (including maltase, α-dextrinase, and sucrase), plays a significant role in carbohydrate metabolism. This enzyme hydrolyzes α-1,4-glycosidic bonds releasing glucose from the non-reducing end of polysaccharides. Starch and other carbohydrates are first broken down by α-amylase into maltose, which is then further hydrolyzed by α-glucosidase into glucose, which is absorbed in the intestine (Vocadlo & Davies, 2008). One effective strategy for managing postprandial hyperglycemia in type 2 diabetes mellitus (T2DM) involves inhibiting the digestion of dietary carbohydrates. By inhibiting both α-amylase and α-glucosidase, it is possible to slow down carbohydrate digestion, delay glucose absorption, and thereby lower blood sugar levels (Kajaria et al., 2013).

Clinically, a variety of drugs are available for managing blood glucose levels, including insulin secretagogues such as sulfonylureas (e.g., gliclazide, glimepiride, glyburide) and non-sulfonylureas (e.g., repaglinide, nateglinide), biguanides like metformin, thiazolidinediones (e.g., rosiglitazone, pioglitazone), intestinal lipase inhibitors (e.g., orlistat), α-glucosidase inhibitors (e.g., acarbose, miglitol, voglibose) (Cheng & Fantus, 2005; Kleinberger & Pollin, 2015) and gliptins, also known as DPP-4 inhibitors (Dipeptidyl Peptidase-4 inhibitors) which work by inhibiting the enzyme DPP-4, leading to an increase in the levels of incretin hormones (Florentin et al., 2022; Karagiannis et al., 2014). However, these medications often come with high costs and a range of side effects. These adverse effects have spurred researchers to explore natural products as alternative sources for digestive enzyme inhibitors, aiming for treatments with fewer and milder side effects (Modak et al., 2007; Shai et al., 2010).

Plants, microbes, and marine sources have long been primary sources of drug discovery. Their inherent bioactivity stems from evolutionary functions, often interacting with mammalian receptors. Unlike synthetic drugs, natural products often defy Lipinski's "Rule of Five" due to unique transport mechanisms and bioactive structures (Beutler, 2019). The development of non-insulin therapies for T2DM has increasingly leveraged natural products and their derivatives.

Numerous antidiabetic drugs are derived from natural sources or inspired by natural compounds (Mukherjee et al., 2006). Metformin, a first-line treatment for T2DM, originates from the plant *Galega officinalis*. Berberine, from *Berberis* species, and *Salacia* extract, from *Salacia reticulata*, provide glucose-lowering effects. Similarly, plant-based compounds like stevioside from *Stevia rebaudiana* and epicatechin from *Pterocarpus marsupium* aid in managing blood sugar. Microbial sources have also contributed significantly. Thus, acarbose, miglitol, and voglibose are alpha-glucosidase inhibitors derived from or inspired by microorganisms like *Actinoplanes* and *Streptomyces*. Insulin, originally sourced from the animal pancreas, was among the earliest examples of natural origin. Exenatide, a GLP-1 receptor agonist, is modeled after exendin-4 from the *Gila monster*'s saliva, and SGLT2 inhibitors, like dapagliflozin, are synthetic derivatives of phlorizin, a compound from apple tree bark (Newman, 2022; Omoirri et al., 2018; Salehi et al., 2019).

Many synthetic diabetes drugs trace their origins to natural processes or structures. Sulfonylureas, while fully synthetic, were developed through studies on sulfur-containing natural molecules. DPP-4 inhibitors (gliptins) were inspired by the incretin system and GLP-1 biology, while TZDs (e.g., pioglitazone) target PPAR-γ, a nuclear receptor activated by natural fatty acids. Modern GLP-1 receptor agonists, such as liraglutide and semaglutide, are synthetic analogs of human incretin hormones. Alpha-glucosidase inhibitors exemplify nature's role in drug discovery, with acarbose and miglitol originating from microbial sources. Even in recombinant insulin production and combination therapies (e.g., metformin with DPP-4 inhibitors), natural products and processes remain pivotal, showcasing the profound influence of nature on diabetes treatment advancements (Cordell & Colvard, 2012; Newman & Cragg, 2012, 2016).

Although a vast array of bioactive secondary metabolites is present in different plant species, only a fraction has been investigated for their significant therapeutic potential (Newman & Cragg, 2016). Initial screenings of medicinal plants using spectral and chromatographic techniques provide fundamental insights into their chemical and pharmacological properties (Juszczak et al., 2019). Recently, fourier-transform infrared (FTIR) spectroscopy and gas chromatography-mass spectrometry (GC-MS) have become standard methods for detecting functional groups and identifying various therapeutic compounds present in medicinal plants (Fan et al., 2018; Satapute et al., 2019). Further, in recent times, computer-aided tools have become crucial in drug discovery, enabling the screening of phytochemicals from medicinal plants (Leelananda & Lindert, 2016). These computational prediction models, also known as predictive tools, play a vital role in guiding pharmaceutical and technological research by forecasting pharmacological, pharmacokinetic, and toxicological properties (Loza-Mejía et al., 2018). Among these tools, molecular docking stands out as an effective and cost-efficient method for drug design and testing. It provides insights into drug-receptor interactions and predicts the binding orientation of drug candidates to their target proteins (Lee & Kim, 2019). This technique facilitates a detailed study by simulating the non-covalent binding of molecules to the active sites of macromolecules, leading to precise predictions of ligand interactions (Bharathi et al., 2014).

Potentilla fulgens Wall ex Sims. (Family: Rosaceae) a medicinal plant used by the Khasi and Jaintia community of Meghalaya, India, locally known as "Lynniang-bru", has been reported to possess anti-hyperglycemic, hypoglycemic, anti-hyperlipidemic, anti-tumor, anti-inflammatory properties, anti-helminthic, and antioxidant activity (Syiem et al., 2009; Syiem et al., 2002). *P. fulgens* contains important phytochemical constituents such as polyphenols, triterpenoids, and flavonoids, which are valued for their therapeutic and commercial potential (Kiran Kaul et al., 2011). *P. fulgens* root extract is traditionally used to address bacterial diseases, diabetes, cancer, and parasitic infections (Rosangkima & Prasad, 2004; Syiem et al., 2002; Tomczyk & Latté, 2009).

A sizeable amount of work has been carried out on this plant. However, most of the studies were focused on using the crude polar extracts of the plant. Given that many phytochemicals of medicinal value cover a broad range of polar and non-polar components, therefore, in this study, we focus on the partial non-polar extract of the plant using column fractionation to identify the different phytochemical constituents present in the extract using Gas-Chromatography Mass Spectrometry (GC-MS). We also evaluated

the extract's efficacy through antioxidant and antidiabetic assays. Furthermore, we conduct in silico molecular docking studies targeting α- amylase and α- glucosidase, which are key enzymes in the initial phase of carbohydrate metabolism.

2. Materials and methods

2.1. Plant material

Roots of *P. fulgens* were collected in July 2021-2022 from Mylliem, Upper Shillong, Meghalaya. A voucher specimen was deposited in the herbarium of the Department of Botany, North Eastern Hill University, Shillong, and identified by Senior taxonomist Dr. P. B. Gurung (voucher number 464).

2.2. Extraction and solvent fractionation

The root samples were separated, weighed, washed, shredded, and shade dried. It was then powdered, homogenized, and repeatedly extracted with 10 vol. of aqueous-methanol solution (1:4) (Harborne, 1998). The extract was concentrated using a roto-evaporator and then lyophilized to get the crude methanol extract (ME). ME (100g) was then chromatographed on silica gel in a 30 X 2.6 (cm) glass column. Elution was carried out using a step-wise gradient, comprising of $CHCl_3$-MeOH (8:2, 7:3, 6:4, 5.5 v/v; 1 l) ratio (Sarker & Nahar, 2007). Different fractions were collected sequentially in beakers over a period based on the color differences. Three major colored fractions were obtained in the order pink followed by yellow and red. Similar colored fractions were collected pooled separately, and dried at room temperature. The dried fractions were then weighed and stored under refrigeration for further use. The fractions (yellow fraction) that tested positive antioxidant activity were selected and referred to herein as NPFE.

2.3. DPPH radical scavenging activity

The DPPH radical scavenging method measured antioxidant activity following the method of Braca et al. (2001) with minor modifications. NPFE at 100 µg/ml concentrations was added to 3 ml of 0.004% DPPH solution. In the control sample, methanol was added in place of the test extract/fraction. The DPPH is violet in color at room temperature which changes to yellow by antioxidants. Absorbance was measured at λ517 nm after 30 min incubation. The percent inhibition was calculated using the equation given below.

$$Inhibition\ (\%) = \frac{Abs517\ (Control) - Abs517\ (Extract)}{Abs517(Control)} x\ 100$$

The inhibitory concentration (IC_{50}) of the sample was calculated using regression analysis from a graph plotting scavenging activity against concentration. The assays were carried out in triplicate.

2.4. Assay for α-amylase inhibitory activity

The assay mixture was prepared by combining 200 µl of 0.02 M sodium phosphate buffer with 20 µl of enzyme and NPFE at concentrations ranging from 20 to 100 µg/ml. After incubating the mixture for 10 min at room temperature, 200 µl of starch was added to each test tube. The reaction was terminated by adding 400 µl of DNS reagent, followed by a 5-minute incubation in a boiling water bath. After cooling, the mixture was diluted with 15 ml of distilled water, and the absorbance was measured at λ540 nm. Control samples were prepared without NPFE. The percentage inhibition was calculated using the formula of Telagari and Hullatti (2015).

$$Inhibition\ (\%) = \frac{Abs540\ (Control) - Abs540\ (Extract)}{Abs540(Control)} x\ 100$$

The IC_{50} values were determined from plots of percent inhibition versus log inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was used as the reference α-amylase inhibitor. All tests were performed in triplicate.

2.5. Assay for α-glucosidase inhibitory activity

P-nitrophenyl-α-D-glucopyranoside, acarbose, and yeast α-glucosidase were purchased from Sisco Research Laboratories (SRL), India. The assay for glucosidase activity was carried out as per the method given by Elya et al. (2012) with certain modifications. The yeast αglucosidase was dissolved in 100 mM phosphate buffer pH 6.8 and was used as the enzyme extract. P-nitrophenyl-α-D-glucopyranoside was used as the substrate. NPFE was used in the concentration ranging from 20-100 µg/ml. Different concentrations of NPFE were mixed with 320 µl of 100 mM phosphate buffer pH 6.8 at 30 °C for 5 min. 3 ml of 50 mM sodium hydroxide was added to the mixture and the absorbance was read at 410 nm. The control samples were prepared without any NPFE. The % inhibition was calculated according to the formula below.

$$Inhibition\ (\%) = \frac{Abs517\ (Control) - Abs517\ (Extract)}{Abs517(Control)} x\ 100$$

The IC_{50} values were determined from plots of percent inhibition versus inhibitor concentration and were calculated by nonlinear regression analysis from the mean inhibitory values. Acarbose was used as the reference α-glucosidase inhibitor. All tests were performed in triplicate.

2.6. Experimental animals

Healthy adult male Swiss albino mice, approximately 4 months old and weighing between 25 and 30 grams were procured from Pasteur Institute Shillong. The study was approved by the Institutional Ethics Committee (NEHU, 31-05-2023) (No. F. 1-182/ZOO/IEC) (Animal Models)/72. The mice were housed under controlled conditions with a temperature maintained between 22 °C and 25 °C, on a 12-hour light/dark cycle. They were provided with a balanced diet obtained from Amrut Laboratory, Pune, India.

2.6.1. Toxicity study

Overnight-fasted male Swiss albino mice (25–30 g) were used for the study. The animals were divided into four groups of six animals each. Groups A to D received intraperitoneal 250, 500, 1000, and 2000, mg/kg of the fractionated extract respectively (limit test as per OECD guidelines) (OECD, 2001) while the control (group E), received distilled water by the same route. General symptoms of toxicity and mortality in each group were observed within 24 hours. Animals that survived after 24 h were observed for any signs of delayed toxicity for 2 weeks.

2.6.2. Administration of NPFE to normal mice

NPFE was administered to normoglycemic mice in varying doses ranging from 150 to 1000 mg/kg body weight via intraperitoneal injection to determine the optimal dose (Syiem et al., 2002). Each treatment group consisted of at least six mice (n = 6). Glucose levels were monitored at various time points for up to 5 days post-administration. The control group received only 2% ethanol, the

solvent used for preparing the extract (Syiem et al., 2002). During the test period, which did not exceed 24 hours, food was withheld but water was provided ad libitum. Food intake, fluid intake, and body weight were recorded for 4 weeks following the extract administration.

2.6.3. Preparation of diabetic mice

Alloxan monohydrate (150 mg/kg body weight) dissolved in acetate buffer (0.15 M, pH 4.5) was administered intraperitoneally to the mice. The control group received only the acetate buffer. Before treatment, the mice were fasted overnight but had access to water ad libitum. Following administration, the animals were monitored for one week, after which their blood glucose levels were measured. Mice exhibiting a blood glucose increase of more than 3–4 times the normal level were classified as diabetic and were used for subsequent tests (Syiem et al., 2002).

2.6.4. Administration of extract to alloxan-induced diabetic mice

Alloxan-induced diabetic mice (n = 6) were administered NPFE intraperitoneally at the optimized dose established with the test conducted with normal mice every alternate day for 3 weeks.

2.6.5. Glucose tolerance test

For the Intraperitoneal Glucose Tolerance Test (IPGTT), animals were divided into four experimental groups and one control group, with each group consisting of at least six mice (n = 6). Both normal and alloxan-induced diabetic mice were fasted overnight but had free access to water. Test samples were administered intraperitoneally to the mice 1.5 hours before they received an oral glucose load of 2 g/kg body weight (Syiem et al., 2002). Glucose concentrations were measured before the glucose administration and then at 30, 60, and 120 min following the glucose load. The control group received only the glucose load without any prior treatment.

Group A: Normal control (NC), received 2% of vehicle
Group B: Diabetic control (DC), received 2% of the vehicle
Group C: Diabetic mice treated with the effective optimum dose of NPFE (250 mg/kg b.w. dose) (Corcoran & Jacobs, 2023; Garber et al., 1997; Sanchez-Rangel & Inzucchi, 2017)
Group D: Diabetic mice treated with the effective optimum dose of metformin (10 mg/kg b.w. dose)
Group E: Diabetic mice treated with an effective optimum dose of vildagliptin (10 mg/kg b.w. dose) (Zhang et al., 2019)
Group F: Diabetic mice treated with an effective optimum dose of Insulin (10 U/kg b.w.) (Syiem et al., 2002)

2.6.6. Statistical analysis

Student's t-tests were used to determine the levels of significance between the control and the test values. Results are expressed as mean ± S.E.M (Standard Error Mean).

2.7. FTIR analysis

The fractionated extract was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in an FTIR spectroscope (Nicolet iS10, Thermo Scientific, USA), with a scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.8. GC/MS Analysis

The GC-MS analysis of each sample was carried out on Shimadzu (GC-2030) series GC-MS equipped with Headspace (HS-20) & QQQ Mass spectrometer GC-TQ8040NX. The column used was SH-Rxi-5 SILMS (0.25 X 30 X 0.25). Helium was used as the carrier gas with a flow rate of 1.00 ml/min. The column temperature was initially set to 50 °C and held for 4 minutes, then increased to 150 °C at a rate of 0.7 °C per minute and held for 4 minutes. The temperature was finally raised to 260 °C at the same rate of 0.7 °C per minute and held for 4 minutes, using a split ratio of 30:70. The injector temperature was 260 °C, the ion source temperature was 220 °C and the interface temperature was 270 °C. The sample was diluted in ethyl acetate 10:100 v/v and 2.0 µl injected with a constant temperature of 260 °C through an autosampler injector. The ionization energy was 70 eV and the mass range of 40–500 AMU.

The management of the GC-MS system, parameter settings for GC and mass spectrometry, and data receipt and processing were performed using Shimadzu Real-time Analysis. The compounds were identified by using the NIST library (Abadie et al., 2022).

2.9. Molecular docking analysis

Molecular docking is used to find the best-fit orientation of ligands and proteins (Morris et al., 2009). The bioactive compounds from fractionated extract were selected for molecular docking analysis. The target proteins α-amylase and α-glucosidase were docked using selected bioactive compounds using BIOVIA, Discovery Studio (version 2021), and AutoDock Vina software, and binding energies were calculated. The ligands and the target proteins were prepared following the standard procedure of protein and ligand preparation and the files were submitted to AutoDock Vina. The binding energy and binding contacts of each ligand were obtained, and the docked data were analyzed using Discovery Studio Visualizer (Ayodele et al., 2023; Guarimata et al., 2023).

2.9.1. Selection of ligands

Ligands (catechol; phenol, 4- propyl; 2,4-Di-tert-butylphenol; 1,3-benzenediol,4-propyl; 1-heptadecene; benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-, methyl ester; dibutyl phthalate; metformin; and acarbose) were obtained from the PubChem drug databases and docked against pancreatic α-amylase (2QV4) and α-glucosidase (5kzw).

2.9.2. Ligand preparation

The 3-D structure of inhibitors with their respective PubChem CID were redeemed and saved in Structure Data File (SDF) format. Furthermore, ligand preparations were continued by taking the 3-D structure of all the ligands and were introduced in Pymol software for conversion of 3-D structure from SDF to Protein Data Bank (PDB) format. Using Pymol software, metals were also removed from the ligand structure for an appropriate docking study. The prepared ligands were saved in PDB format for further docking studies.

2.9.3. Protein preparation

The present study is an expressive-analytical study and the 3D structure of target proteins of α-amylase and α-glucosidase available from the PDB database (https://www.rcsb.org/) was used. Two target proteins were used in this study viz. (i) PDB ID: 2QV4 (α-amylase), (ii) PDB ID: 5KZW (α-glucosidase) and their three-dimensional structures were retrieved from PDB (Protein Data

Bank). These acquired proteins were imported to clean and prepare the protein using Pymol software. All the water molecules were removed from the crystal structure of the protein. The Pymol software was used for protein preparation. Polar hydrogens were added to the structure and Kollmann charges were applied. Further, missing residues in the protein structure were added during the protein preparation. The protein structures were visualized on PyMOL 1.3 or 2.5 by demarcating the transmembrane regions if any, present in the protein. While the pdbqt files were generated for the proteins to initiate the grid parameters, we considered 1Å and x, y, and z coordinates to establish the size of the protein for docking studies. MGL tool 1.5.7 was used for generating necessary protein and ligand files along with the respective grids and dock files and the grid generation and docking were subsequently executed through AutoGrid 4.0 and AutoDock 4.0 respectively.

2.9.4. Molecular docking

Docking was performed by the Pyrx Software program, using the implemented empirical free energy function. In all dockings, a grid map with 50 X 57 X 43 points and a grid-point spacing of 1.000 Å was applied. The best conformation with the lowest docked energy was chosen from the docking search. The interactions of complex protein-ligand conformations including hydrogen bonds and bond lengths were analyzed using Pymol software, UCSF Chimera, and Accelrys Discovery Studio Visualizer software.

3. Results and discussion

3.1. DPPH scavenging activity

The extract exhibited a concentration-dependent antioxidant activity. Ascorbic acid which is a well-known antioxidant showed a higher degree of free radical scavenging assay than that of the plant extract at each concentration point. The ability of the fractionated plant extract to scavenge the DPPH free radical was assessed and results were expressed in terms of % inhibition (Figure 1) and IC_{50} values (Table 1). A lower IC_{50} corresponds to a more effective free radical lowering potential. The IC_{50} of NPFE was found to be 43.812 µg/ml, while the IC_{50} of the standard reference ascorbic acid was found to be 22.541 ug/ml which is in the range of previously reported study by Hossain et al. (2017), with IC_{50} value of 34.88 µg/ml. Thus, NPFE has a lower antioxidant potential than ascorbic acid. However, this has to be viewed from the context that the fraction contains a wide range of chemical entities with varying concentrations much lower than the concentration of the standard used thereby exhibiting lower IC_{50} than the purified reference.

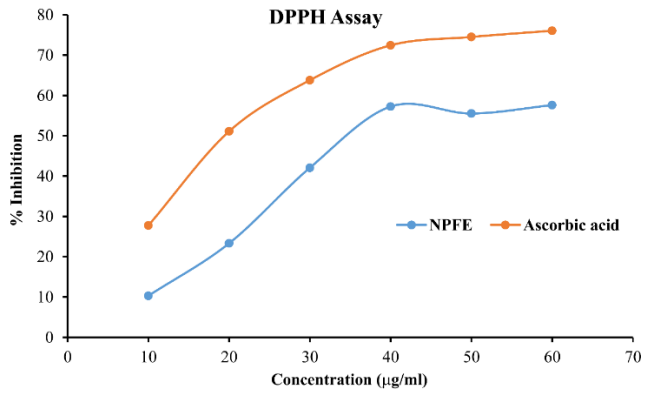


Figure 1. DPPH assay of NPFE showing % inhibition as a function of concentration
(NPFE: Non- Polar Fractionated Extract)

Table 1. IC_{50} values of NPFE and ascorbic acid

Sl. No	Sample	DPPH IC_{50} (µg/ml)
1	NPFE	43.812 ± 1.789
2	Ascorbic acid	22.541 ± 2.168

Results are expressed as mean± SEM

3.2. In vitro α-amylase and α-glucosidase inhibition activity

The inhibitory activity of NPFE against the digestive enzymes α-amylase and α-glucosidase were evaluated in comparison to the standard drug acarbose (Figures 2 and 3). The IC_{50} value for α-amylase and α-glucosidase inhibition of NPFE was found to be 44.064 µg/ml and 38.46 µg/ml respectively (Table 2). When compared to values reported for other plant extract (Shrestha et al., 2022), NFPE was found to be significantly a more potent inhibitor. However, in vitro α-amylase and α-glucosidase activity assay showed that the NPFE fraction could inhibit the enzyme at IC_{50} value higher than the standard drug acarbose with an IC_{50} 39.290 ug/ml and 17.077 ug/ml, respectively, indicating their slightly lower efficacy as the fractionated components contains only non-polar entities thereby reducing their overall potential. Further, the active chemical entity contributing to the inhibitory action may be present in a lower amount as mentioned above and therefore not comparable to the purified reference drug acarbose.

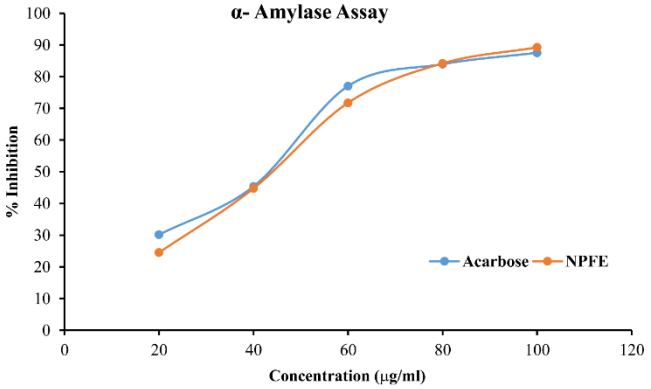


Figure 2. α-Amylase inhibitory assay of NPFE and the reference standard acarbose showing % inhibition as a function of concentration
(NPFE: Non-polar fractionated extract)

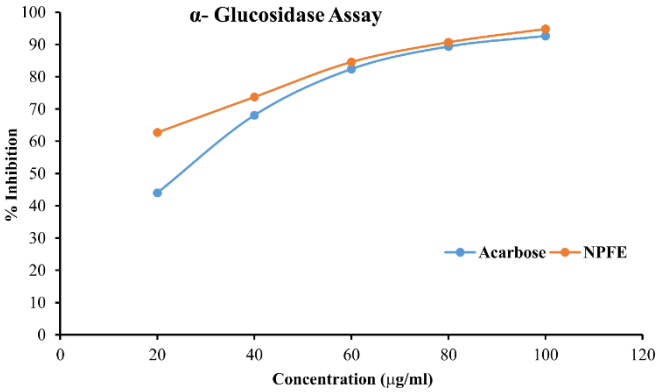


Figure 3. α-Glucosidase inhibitory assay of NPFE and the reference standard acarbose showing % inhibition as a function of concentration
(NPFE: Non-polar fractionated extract)

Table 2. IC_{50} value of NPFE against α amylase and α-glucosidase

Sample	α-Amylase (µg/ml)	α-Glucosidase (µg/ml)
NPFE	44.064 ± 3.137	38.461 ± 1.177
Acarbose	39.290 ± 3.799	17.077 ± 2.279

P. fulgens plays a significant role in traditional medicine, particularly for its anti-diabetic properties. The methanolic extract of its root has been shown to exhibit both hypoglycaemic and anti-hyperglycaemic effects in alloxan-induced diabetic mice (Syiem et al., 2002). It is pertinent to note that the inhibition of α-glucosidase has been identified as a key mechanism behind the anti-hyperglycaemic effects of various plant extracts and their isolated compounds. (Li et al., 2005; Patel & Mishra, 2012; Shim et al., 2003). Inhibiting this enzyme prevents the rise in blood glucose levels that typically follow a meal. α-Amylase breaks down complex polysaccharides into oligo-saccharides, and then intestinal α-glucosidase catalyzes the final step, releasing absorbable carbohydrates. (Fatmawati et al., 2011; Holman et al., 1999; Patel & Mishra, 2012; Tewari et al., 2003; Van de Laar et al., 2005).

3.3. Toxicity test

After the acute toxicity studies, no mortality was observed up to the dose of 2000 mg/kg for the NPFA. The extract was considered to be safe as per the limit test.

3.4. Study on effect of NPFE on blood glucose level of normoglycemic and diabetic mice

Test extract was administered intraperitoneally to male Swiss albino mice and their effects were studied for a period of up to 24 hours. The effect of the extract was first assessed in normoglycemic mice. The dose found to be optimum (i.e., the lowest dose with the highest blood glucose lowering potential) was then used to administer to alloxan-induced diabetic mice in order to assess the plant's antihyperglycemic activity.

The effect on blood glucose levels in normoglycemic mice after the administration of the fractionated extract (NPFE) at varying doses exhibited a reduction in blood glucose level in a dose- and time-dependent manner wherein all the doses were able to bring down blood glucose levels to varying degrees. The optimum dose was found to be 250 mg/kg b.w. which was the minimal dose that could lower blood glucose levels at all time intervals studied (Figure 4).

3.5. Intraperitoneal glucose tolerance tests (IPGTT) in normoglycemic and diabetic mice

The IPGTT is a gold standard for assessing the efficacy of antidiabetic drugs (American Diabetes Association, 2022; WHO, 1999). The test was performed in both normoglycemic and alloxan-induced diabetic mice to check the effect of the plant fractionated extract (NPFE) on glucose tolerance and to assess the ability of the NPFE to decrease the glucose peak after the oral glucose load. In addition, the standard drugs metformin (a biguanide), vildagliptin (DPPIV Inhibitor), and insulin were used to provide insight into the pattern of action of the fractionated extract.

Control normoglycemic mice challenged with a glucose load of 2 g/kg b.w. showed an elevation in blood glucose at 30 mins which gradually declined as time progressed. This glucose peak was significantly decreased by 44% ($p < 0.01$) in extract-treated mice. Notably, the extract was more efficient than metformin in suppressing the glucose peak at 30 min. The extract continued to exert its effects at 1 and 2 hours (60 and 120 mins) and was effective even at 24 hours (1440 min). The overall pattern of glucose tolerance was seen to resemble that of vildagliptin concerning the magnitude and pattern of glucose suppression (Figures 5 and 6).

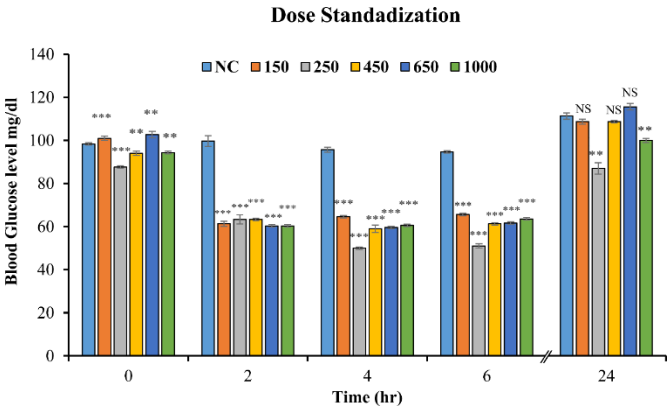


Figure 4. The effect of varying doses (150, 250, 450, 650, and 1000mg/kg b.w.) of NPFE on the blood glucose of normoglycemic mice was measured at different time intervals

Values are expressed as mMean ± SEM. SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ represent the level of significance against the normal control. NS stands for not significant). NC: Normal control. NPFE: Non-polar fractionated extract

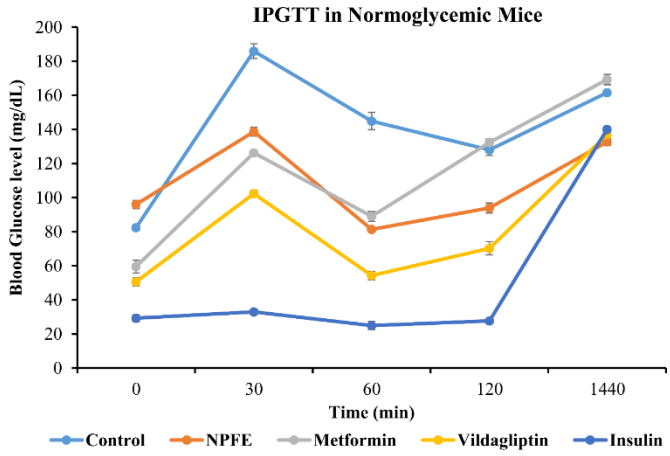


Figure 5. Glucose tolerance in normoglycemic mice administered with 250 mg/kg b.w. of NPFE and reference drugs

Values are expressed as mean ± SEM. NPFE: Non-polar fractionated extract

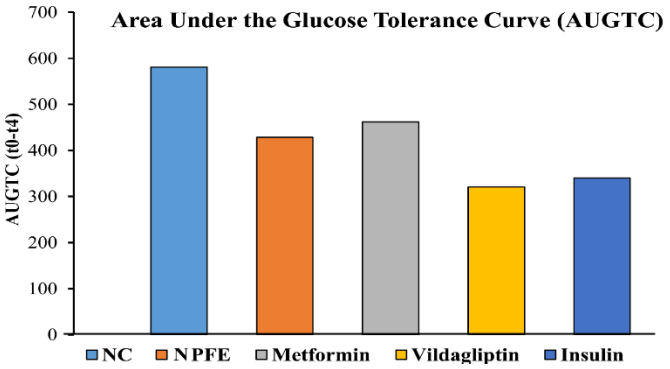


Figure 6. The area under the curve of the intraperitoneal glucose tolerance in NC, NPFE, metformin, vildagliptin, and insulin

(NPFE: Non-polar fractionated extract)

At the height of the glucose peak, the extract exhibits glucose-lowering properties by reducing the blood glucose level by 44%. The

magnitude of reduction was observed to be highest with insulin (83%), followed by vildagliptin (63%), extract (44%), and metformin (38%).

The same pattern was also observed in diabetic mice (Figures 7 and 8). Here, the extract exhibited glucose-lowering properties by reducing the blood glucose level by 68%, with the magnitude of reduction being highest in insulin at 92%, followed by vildagliptin at 75%, the fractionated extract at 68%, and metformin at 51%.

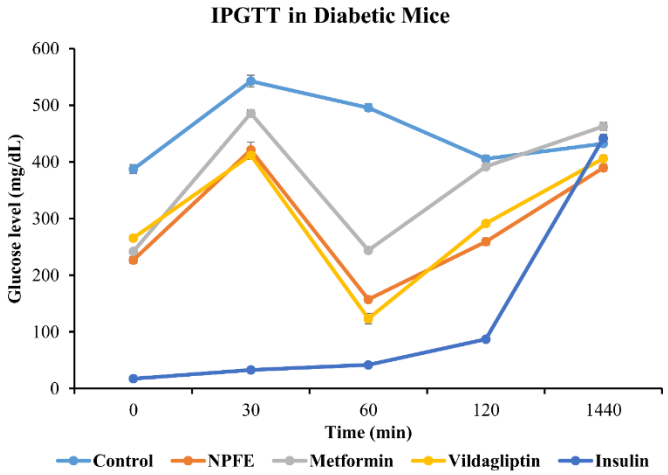


Figure 7. Glucose tolerance in alloxan-induced diabetic mice administered with 250 mg/kg b.w. of NPFE and reference drugs
Values are expressed as mean ± SEM. NPFE: Non-polar fractionated extract

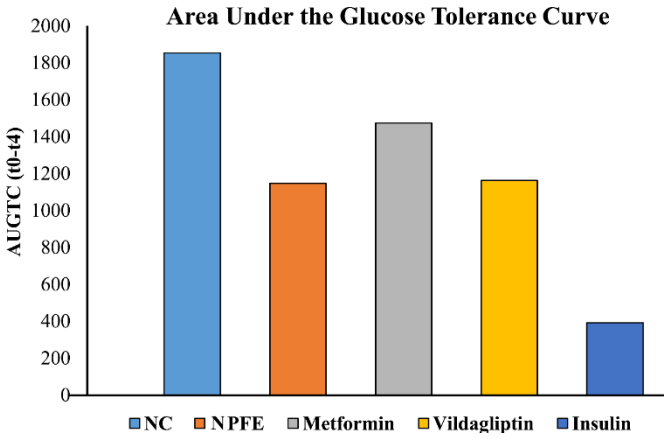


Figure 8. The area under the curve of the intraperitoneal glucose tolerance in NC, NPFE, metformin, vildagliptin, and insulin

Table 3. Functional groups present in NPFE-analysis from FTIR

Frequency range (cm^{-1})	Sample wave number (cm^{-1})	Functional group
3870-3550	-	O-H stretch alcohol
3500-3200	3424.01	O-H stretch vibration presence of alcohols, phenols
3300-2850	2944.29	O-H stretch vibration, carboxylic acids
2500-2300	2354.97	C-H stretch vibration, alkenes
2260-2100	-	C=C stretch vibration, alkynes
1990-1739	-	Ester C=O stretch, lipid, triglycerides
1700-1600	1618.23	C=C stretch vibration, alkenes
1550-1475	1518.46	N-O asymmetric stretch, nitro compounds
1470-1400	1451.20	C-C stretch vibration, aromatics
1400-1320	1383.25	N-O stretch vibration, nitro compounds
1300-1290	-	C-O stretch vibration, alcohol, carboxylic acids, esters, ether
1275-1150	1245.25	C-H wag stretch vibration, alkyl halides
1020-1000	-	C-N stretch vibration, aliphatic amines
990-800	978.96, 921.90, 866.48, 821.82	N-H wag stretch vibration, primary & secondary amines
790-690	779.33, 700.99	C (triple bond) C-HC-H bend stretch vibration, alkynes
680-510	669.53	C-Br stretch vibration, alkyl halides, glycogen
490-400	-	Halogen compound

Thus, it may be inferred from the study on normal and diabetic mice that the nonpolar fractions have significant glucose-lowering properties which may contribute synergistically to the antidiabetic property reported for the polar extracts (Syiem et al., 2002).

3.6. FTIR analysis

FTIR spectroscopy was employed to identify the functional groups of bioactive components present in NPFE (Figure 9, Table 3). FTIR spectrum confirmed the presence of hydroxyl group (O–H), aldehyde (C–H), nitrogen-containing group (N–O), alkanes (C–C), amines (N–H), alkynes (C≡C), aliphatic bromo compounds (C–Br), phenols, carboxylic acids, glycogen, alkyl halides, halogen, aliphatic amines, primary and secondary amines, esters, ether, aromatics, lipids, triglycerides, nitro compounds and these functional groups are the integral parts of various secondary metabolites such as alkaloids, flavonoids, terpenoids, polyphenol and tannins.

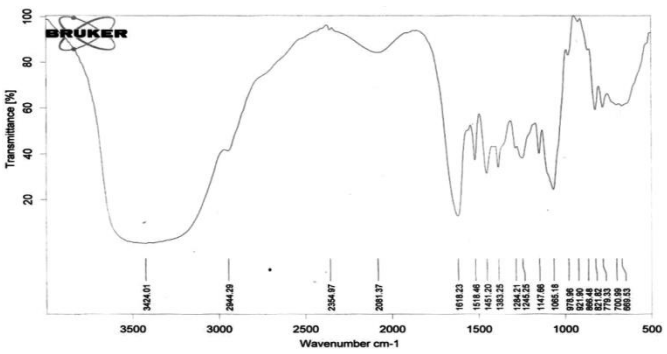


Figure 9. FTIR spectrum of NPFE

3.7. Gas chromatography-mass spectrometry (GC–MS) analysis

The GC–MS chromatogram of the fractionated root extract of *P. fulgens* (NPFE) recorded a total of 22 peaks (Figure 10) corresponding to the bioactive compounds that were recognized by relating their peak retention time, peak area (%), height (%) and mass spectral fragmentation patterns to that of the known compounds described by the National Institute of Standards and Technology (NIST) library. The molecular formula of 22 phytocompounds identified in the extract is presented in Table 4 along with their retention time.

Table 4. Retention time (min), molecular weight, molecular formula, structure of phytochemical constituents identified from NPFE using gas chromatography-mass spectrometry, and their possible medicinal uses

Sl. No.	Retention time (min)	Molecular weight	Molecular formula	Name of the compound	Medicinal Uses
1	5.546	156	$C_{11}H_{24}$	Undecane	Anti-allergic and anti-inflammatory (Choi et al., 2020)
2	6.898	110	$C_6H_6O_2$	Catechol	Anti-cancer, anti-inflammatory, antioxidant, anti-diabetic, antimicrobial, antiviral, and antifungal properties (Surana et al., 2023)
3	8.594	136	$C_9H_{12}O$	Phenol, 4-propyl-	Ecological fungicide (Sun et al., 2024)
4	9.462	206	$C14_6H_{22}O$	2,4-Di-tert-butyl-phenol	Anticancer, antifungal, antihelminthic, rich in antioxidant, antiviral, antibacterial (Aravinth et al., 2023)
5	10.066	362	$C_{18}H_{35}BrO_2$	2-Bromopropionic acid, pentadecyl ester	Identified in many plants but medicinal properties remain unknown
6	10.611	152	$C_9H_{12}O_2$	1,3-Benzenediol, 4-propyl-	Antibacterial and anti-quorum sensing (Deryabin & Tolmacheva, 2015)
7	11.581	238	$C_{17}H_{34}$	1-Heptadecene	Identified in many plants but medicinal properties remain unknown
8	12.698	292	$C_{18}H_{28}O_3$	Benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-,methyl ester	Antibacterial activity (Alqahtani et al., 2020)
9	12.955	364	$C_{26}H_{52}$	1-Hexacosene	Analgesic and anti-inflammatory activity (Kariuki et al., 2012)
10	14.210	364	$C_{26}H_{52}$	1-Hexacosene	Analgesic and anti-inflammatory activity (Kariuki et al., 2012)
11	15.230	410	$C_{28}H_{42}O_2$	(1,1'-Biphenyl)-2,3'-diol, 3,4',5,6'-tetrakis(1,1-dimethyl ethyl)	Antimicrobial potential (Yahya Al-Ghamdi, 2022)
12	15.363	396	$C_{24}H_{50}O$	1-*n*-Tetracosanol	Wound healing by expressing inflammatory cytokines and matrix metalloproteinase (Shalini et al., 2023)
13	15.845	312	$C_{20}H_{38}O_3$	Octadecanoic acid, 3-oxo-,ethylester	Identified in many plants but medicinal properties remain unknown
14	15.935	342	$C_{25}H_{42}$	1H-Indene,1-hexadecyl-2,3-dihydro-	Identified in many plants but medicinal properties remain unknown
15	16.047	330	$C_{19}H_{38}O_4$	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	Identified in many plants but medicinal properties remain unknown
16	16.429	410	$C_{28}H_{58}O$	Octacosanol	Cholesterol-lowering and anti-inflammatory effects (Zhou et al., 2022)
17	16.566	470	$C_{27}H_{50}O_6S$	Eicosanoic acid, 2-(acetyloxy)-1-[(acetyloxy) methyl] ethyl ester	Identified in many plants but medicinal properties remain unknown
18	16.972	342	$C_{25}H_{42}$	1H-Indene, 1-hexadecyl-2,3-dihydro-	Identified in many plants but medicinal properties remain unknown
19	17.110	358	$C_{21}H_{42}O_4$	Octadecanoic acid, 2,3-dihydroxypropyl ester	Identified in many plants but medicinal properties remain unknown
20	17.447	394	$C_{22}H_{41}F_3O_2$	Eicosyl trifluoroacetate	Antioxidant activity (Erwin et al., 2018)
21	17.610	470	$C_{27}H_{50}O_6$	Eicosanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl] ethyl ester	Identified in many plants but medicinal properties remain unknown

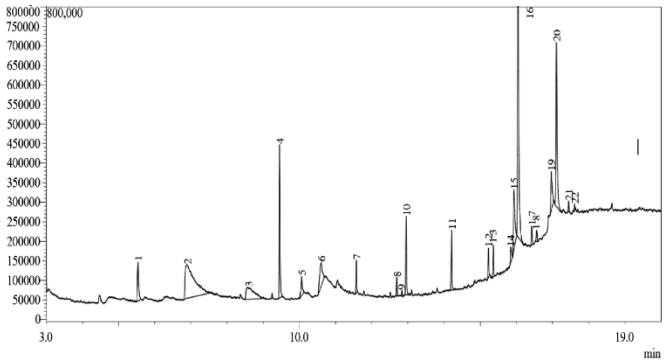


Figure 10. GC-MS of NPFE showing relative intensity and respective retention times

The major components present in NPFE include undecane (3.06 %), catechol (19.15 %), phenol, 4-propyl- (5.88 %), 2,4-di-tert-butylphenol (7.52 %), 2-bromopropionic acid pentadecyl ester (1.61 %), 1,3-benzenediol, 4-propyl (4.14 %), l-heptadecene (1.47 %), benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester (0.80 %), 1-hexacosene (6.37 %), (1,1'-Biphenyl)-2,3'-diol, 3,4',5,6'-tetrakis(1,1-dimethylethyl)- (1.23 %), -1 *n*-tetracosanol (1.47 %), octadecanoic acid, 3-oxo-,ethylester (1.13 %), 1H-indene, 1-hexadecyl-2,3-dihydro- (5.41 %), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethylester (23.45 %), octacosanol (0.85 %), eicosanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl]ethylester (0.61 %), 1H-indene,1-hexadecyl-2,3-dihydro-(3.57 %), octadecanoic acid, 2,3-dihydroxypropylester (11.51 %), eicosyltrifluoroacetate (0.48 %), eicosanoic acid, 2-(acetyloxy)-1-[(acetyloxy) methyl] ethylesters (0.29%).

3.8. Molecular docking

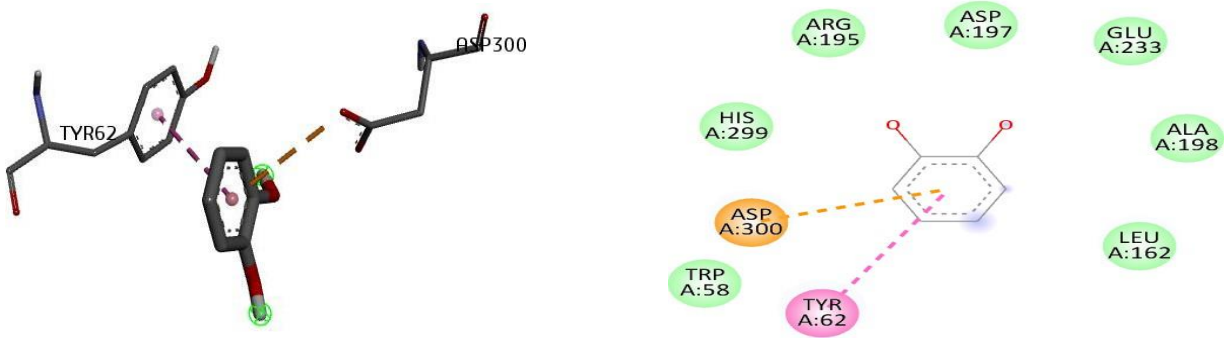
Compounds identified from GC-MS were checked for their drug-likeness, where compounds were selected based on Lipinski's rule of five parameters such as molecular weight, log P, number of hydrogen bond donors, and number of hydrogen bond acceptors **(Table 5)**. Those adhering to Lipinski's rule of five were then used for docking analysis with α-amylase and α-glucosidase. Six compounds (catechol, phenol, 4-propyl-, 2,4-di-tert-butylphenol, 1,3-benzenediol, 4-propyl, benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-, methyl ester, dibutyl phthalate) identified from GC-MS **(Table 4)** complied with the Lipinski's rule and hence were selected for docking **(Figures 11 and 12)**. Metformin and acarbose were used as standard controls. The 2D structures of all selected bioactive compounds were retrieved from the PubChem database.

The binding analysis between α-amylase, α-glucosidase protein, and ligands revealed that the binding pattern varied with the nature of the ligands. The docking analysis of bioactive compounds is shown in **Figures 11** and **12**. The docking results are represented in the form of minimum binding energy values **(Tables 6 and 7)**. Catechol, phenol, 4-propyl, and 1,3-benzenediol, 4-propyl showed better binding affinities (–5.2, –5.9, and –5.9 kcal/mol, respectively) than the standard drugs metformin and acarbose (–5.3 and –7.6 kcal/mol, respectively) when docked with α-amylase **(Figures 11 and Table 6)** while with α-glucosidase all the six compounds showed

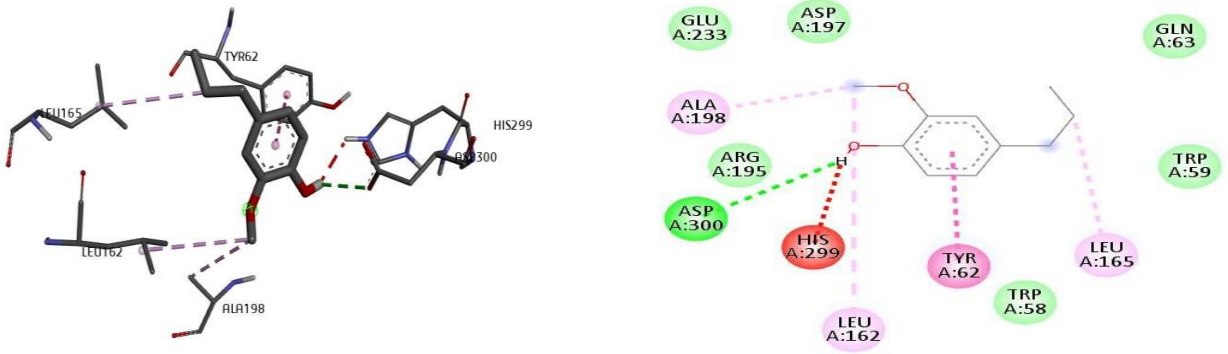
better binding affinity (–5.2, –5.6, –5.9, –5.3, –6.6, and –5.8 kcal/mol) than acarbose (–6.7 kcal/mol) but a comparable binding affinity with metformin (–4.6 kcal/mol) (Figure 12 and Table 7).

Table 5. Phytochemicals identified from GCMS and Lipinski's rule of five adherence

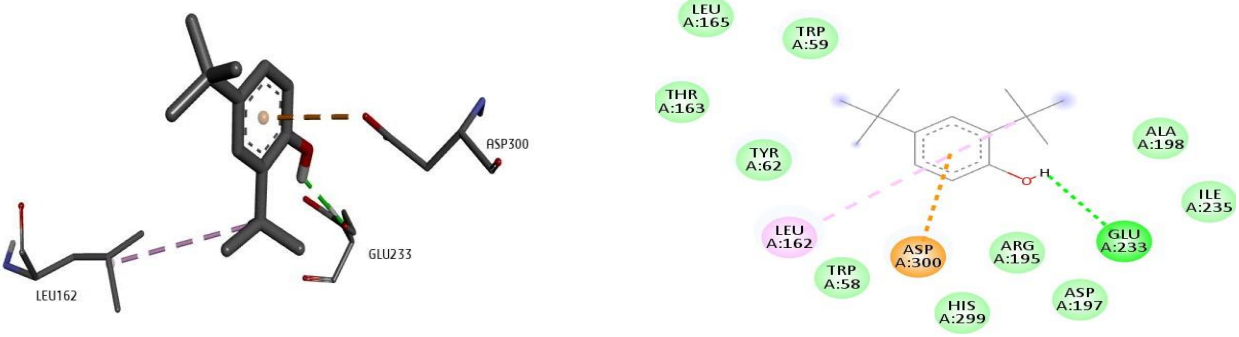
Sl. No.	Name of compound	Mol. Wt.	X LogP3	Hydrogen bond donor	Hydrogen bond acceptor	Rotatable bond
1	Catechol	110.11	0.9	2	2	0
2	Phenol, 4-propyl	136.19	3.2	1	1	2
3	2,4-Di-tert-butyl-phenol	206.32	4.2	1	1	2
4	1,3-Benzenediol, 4-propyl	152.19	1.8	2	2	2
5	Benzenepropanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-methyl ester	292.4	5	1	3	6
6	Dibutyl phthalate	278.34	4.7	0	4	10



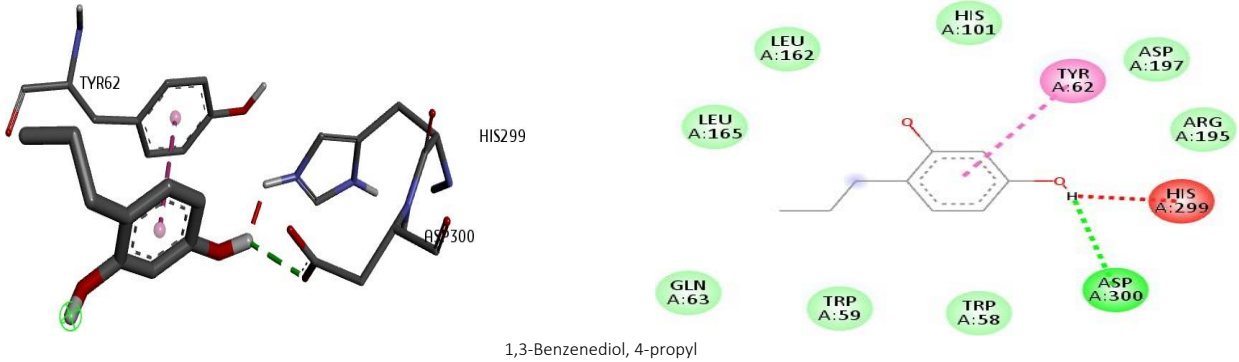
Catechol



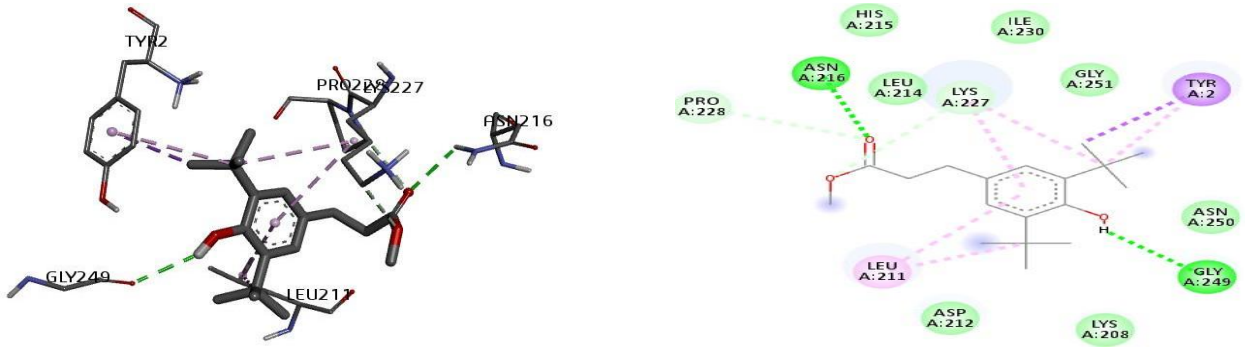
Phenol, 4-propyl



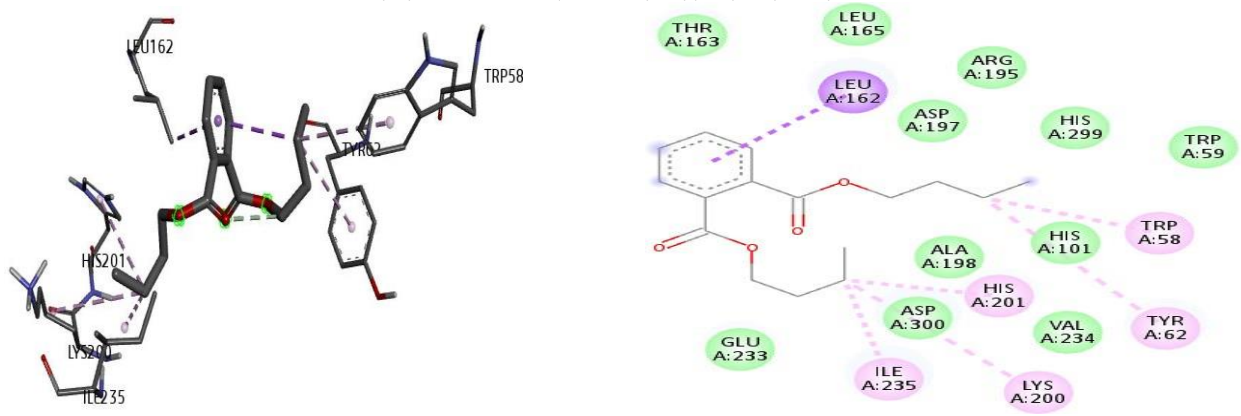
2,4-Di-tert-butylphenol



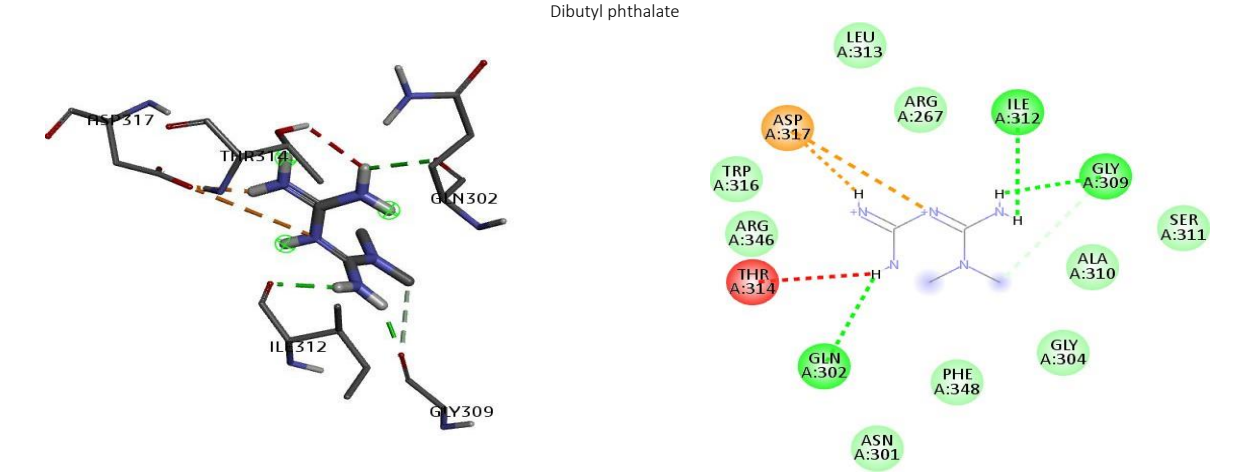
1,3-Benzenediol, 4-propyl



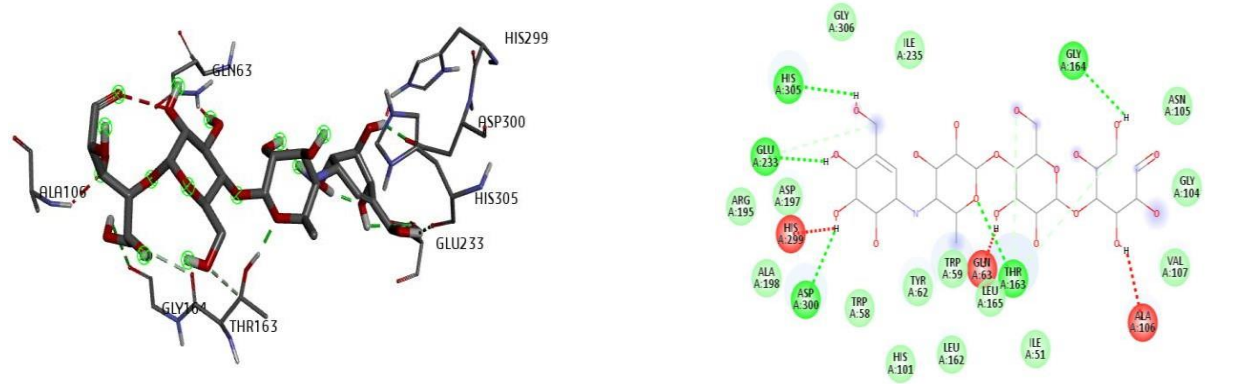
Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-methyl ester



Dibutyl phthalate

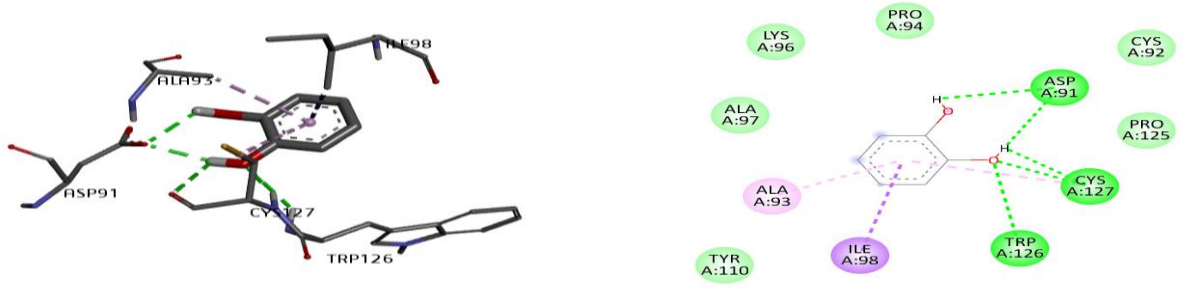


Metformin

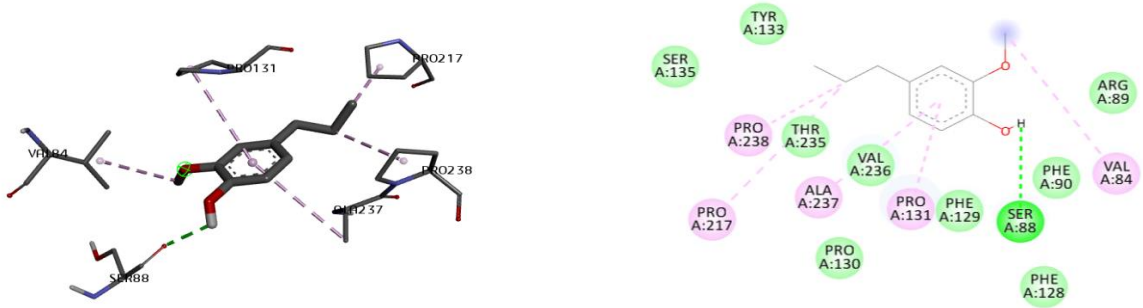


Acarbose

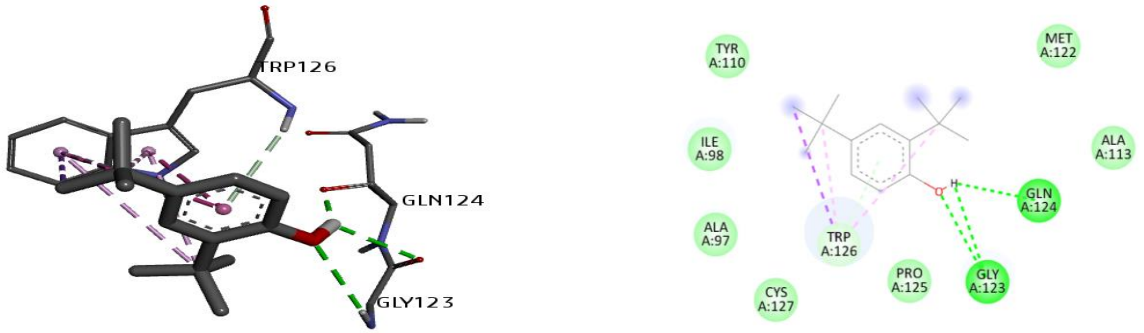
Figure 11. Molecular docking of pancreatic α-amylase (2QV4) binding domain complexed with catechol, phenol, 4-propyl, 2,4-di-tert-butylphenol, 1,3-benzenediol, 4-propyl, benzenepropanoic acid, 3,5-bis (1,1- dimethylethyl)-4-hydroxy-, methyl ester, dibutyl phthalate, metformin, and acarbose.



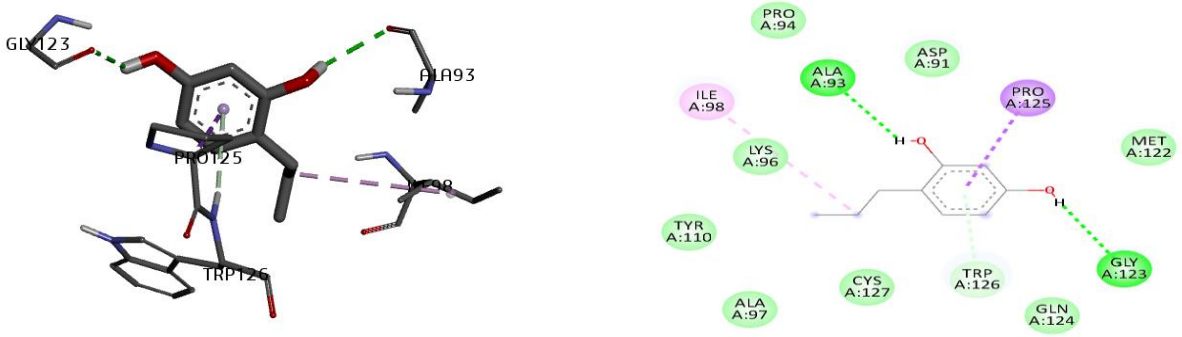
Catechol



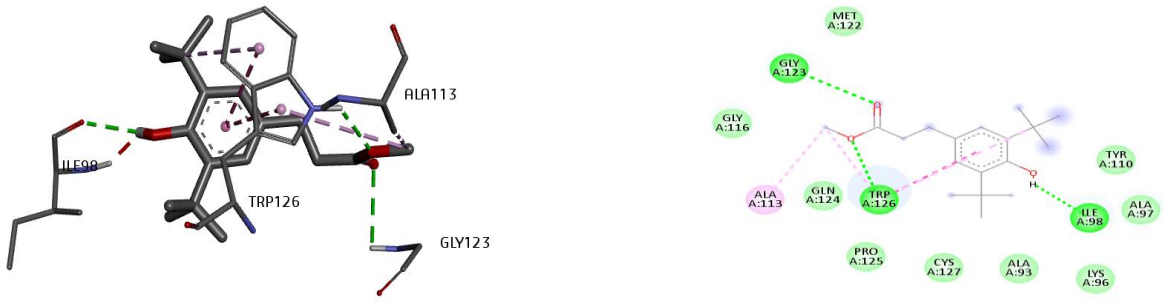
Phenol, 4-propyl



2,4-Di-tert-butylphenol



1,3-Benzenediol, 4-propyl



Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-methyl ester

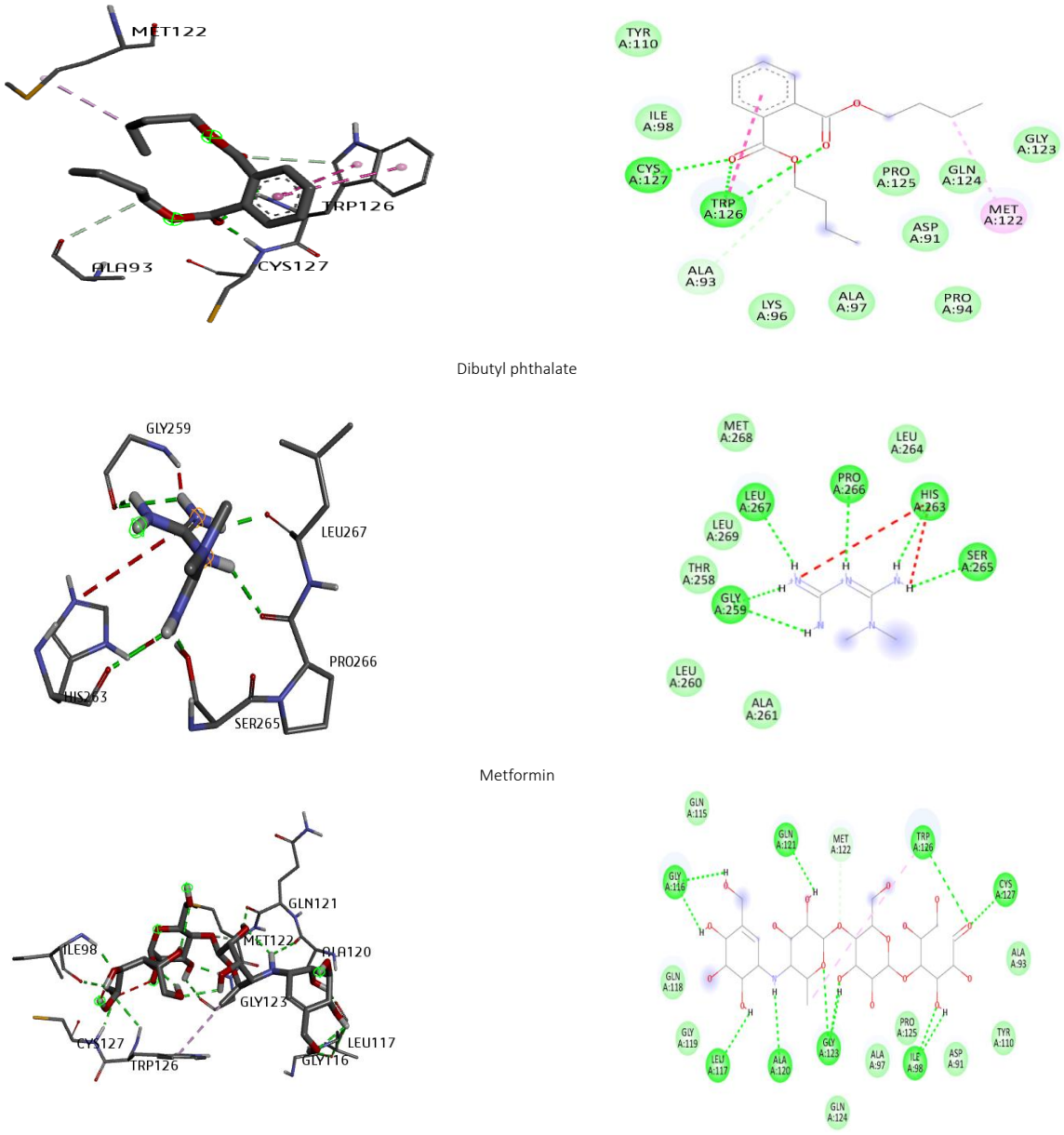


Figure 12. Molecular docking of pancreatic α-glucosidase (5KZW) binding domain complexed with catechol, phenol, 4-propyl, 2,4-di-tert-butylphenol, 1,3-benzenediol, 4-propyl, benzenepropanoic acid, 3,5-bis (1,1- dimethylethyl)-4-hydroxy-, methyl ester, dibutyl phthalate, metformin, and acarbose

Table 6. Molecular docking analysis of phytoconstituents from NPFE and their binding energy with α-amylase (2QV4)

Protein name	Ligand name	Binding energy (kcal/mol)	No. of H-bonds	Interacting residue
2QV4	Catechol	–5.2	(00)	ASP: 300, TRY: 62
	Phenol, 4-propyl	–5.9	(01) HI: 300Å	ASP: 300 (H1), ALA: 198, HIS: 299, TYR: 62, LEU: 162, LEU: 165
	2,4-Di-tert-butylphenol	–6.6	(01) HI: 2.39Å	GLU: 233 (H1), ASP: 300, LEU: 162
	1,3-Benzenediol, 4-propyl	–5.9	(01) H1: 2.49Å	ASP: 300 (H1), HIS: 299, TYR: 62
	Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-methyl ester	–6.7	(02) H1: 2.62Å, H2: 2.60Å	ASN: 216 (H1), GLY: 249 (H2), PRO: 228, LEU: 211, LYS: 227, TYR: 2
	Dibutyl phthalate	–6.2	(00)	ILE: 235, LYS: 200, TYR: 62, TRP: 58, HIS: 201, LEU: 162
	Metformin	–5.3	(03) H1: 2.18Å, H2: 1.94Å, H3: 2.53Å	ILE: 312 (H1), GLY: 309 (H2), GLN: 302 (H3), THR: 314, ASP: 317
	Acarbose	–7.6	(05) H1: 2.73Å, H2: 2.97Å, H3: 2.46Å, H4: 2.73Å, H5: 300Å	HIS: 305 (H1), GLU: 233 (H2), ASP: 300 (H3), THR: 163 (H4), GLY: 164 (H5), HIS: 299, GLN: 63, ALA: 106

Table 7. Molecular docking analysis of phytoconstituents from NPFE and their binding energy with α-glucosidase (5kzw)

Protein name	Ligand name	Binding energy (kcal/mol)	No. of H-bonds	Interacting residue
α-glucosidase	Catechol	–5.2	(03) H1:2.11Å, H2:1.99Å, H3:2.66Å	ASP:91(H1), CYS:127(H2), TRP:126(H3), ILE:98, ALA:93
(5kzw)	Phenol, 4-propyl	–5.6	(01) H1:2.50Å	SER:88(H1), VAL:84, PRO:131, ALA:237, PRO:217, PRO:238
	2,4-Di-tert-butylphenol	–5.9	(02) H1:1.96Å, H2:2.71Å	GLN:124(H1), GLY:123(H2), TRP:126
	1,3-Benzenediol, 4-propyl	–5.3	(02) H1:2.43Å, H2:2.48Å	GLY:123(H1), ALA:93(H2), ILE:98, PRO:125, TRP:126
	Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-methyl ester	–6.6	(03) H1:3.01Å, H2:2.50Å, H3:2.63Å	GLY:123(H1), TRP:126(H2), ILE:98(H3), ALA:113
	Dibutyl phthalate	–5.8	(02) H1:1.79Å, H2:2.27Å	CYS:127(H1), TRP:126(H2), ALA:93, MET:122
	Metformin	–4.6	(05) H1:2.33Å, H2:1.97Å, H3:2.33Å, H4:2.27Å, H5:1.88Å	GLY:259(H1), LEU:267(H2), PRO:266(H3), HIS:263(H4), SER:265(H5)
	Acarbose	–6.7	(08) H1:2.37Å, H2:2.15Å, H3:2.73Å, H4:1.95Å, H5:1.95Å, H6:2.04Å, H7:2.54Å, H8:2.02Å	GLY:116(H1), LEU:117(H2), ALA:120(H3), GLY:123(H4), ILE:98(H5), CYS:127(H6), TRP:126(H7), GLN:121(H8), MET:122

Molecular docking is a crucial drug discovery tool with its strengths such as providing efficient screening and predictive insights. However, it has its limitations such as oversimplified scoring, rigid protein models, and inadequate solvent modeling. Discrepancies arise from neglecting protein flexibility, solvation and desolvation also arise (Meng et al., 2011). Enhancements include combining docking with molecular dynamics simulations, advanced scoring methods, flexible docking algorithms, and experimental validation. Tailoring protocols to biological systems and leveraging complementary approaches improves reliability, facilitating accurate drug discovery and development (Krovat et al., 2005).

4. Conclusions

The above study revealed the antioxidant and antidiabetic potential of the non-polar fractionated extract of *P. fulgens* through its actions on the carbohydrate hydrolyzing enzymes α-amylase and α-glucosidase. The study also showed the presence of phytochemical entities with drug likeness which may contribute to the antidiabetic property through their strong binding as indicated by the docking studies. Therefore, it may be concluded that the nonpolar fraction of the root of this plant contributes to the anti-diabetic potential of the plant and may act synergistically with the polar fractions adding up holistically to the medicinal property of *P. fulgens*.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

Ethical approval for this study was obtained from the Institutional Ethics Committee (NEHU, 31-05-2023) (No. F. 1-182/ZOO/IEC (Animal Models)/72.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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Supplementary File

None.

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