COMPARATIVE EVALUATION OF NEPHROPROTECTIVE POTENTIAL OF ROOT, STEM AND LEAF OF *PLUMBAGO ZEYLANICA*: AN *IN-SILICO* AND *IN-VITRO* APPROACH

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Abstract

Plants have long been recognized as valuable sources of medicine, with numerous herbal drugs derived from plant compounds. *Plumbago zeylanica Linn*., a member of the Plumbaginaceae family, is a perennial sub scandent shrub widely used in traditional Indian medicine. The study aims to explore the potential of various parts of *Plumbago zeylanica* in the treatment of diabetic nephropathy by investigating *in-vitro* experiments and conducting molecular docking studies with proteins associated with the disease. The *in-vitro* findings exhibited that the extract of roots have greater inhibition activity than stem extract, followed by the leaf extracts of *Plumbago zeylanica.* The glucose uptake assay showed that the enhancement of glucose transport was different for all three concentrations of glucose solution. In molecular docking, the highest binding affinities of *Plumbago zeylanica* bioactive compounds and target proteins were ranging from -7.196 to -9.447. Additionally, the lowest binding affinities of *Plumbago zeylanica* bioactive compounds and target proteins were ranging from -3.779 to -7.196, which were also higher than reference compound for different active constituents. ADMET prediction was performed to assess the pharmacokinetic properties of the active constituents. The findings of this study will shed light on the phytochemical composition of *Plumbago zeylanica* and its potential as a therapeutic agent for diabetic nephropathy.

Keywords: Diabetic nephropathy, *Plumbago zeylanica, In-vitro*, Molecular docking, ADME-T Prediction

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DOI: 10.48047/ecb/2023.12.si5a.0596

1. Introduction

Diabetic nephropathy is the leading cause of chronic kidney disease in patients starting renal replacement therapy (1) and is associated with increased cardiovascular mortality (2). Diabetic nephropathy has been classically defined by the presence of proteinuria >0.5 g/24 h. Seminal European studies published in the early 1980s revealed that small amounts of albumin in the urine, undetectable by conventional methods, were predictive of the later development of proteinuria in type 1 (3–5) and type 2 (6) diabetic patients.

Plants play a vital role as valuable sources of medicine, with a considerable number of drugs being derived from them. One such plant commonly used in the traditional Indian system of medicine is Chitraka, scientifically known as *Plumbago zeylanica Linn*. It belongs to the Plumbaginaceae family. *Plumbago zeylanica* is a perennial, sub scandent shrub and is widely found in the wild as well as in cultivation due to its numerous therapeutic benefits (7). The roots of *Plumbago zeylanica* exhibited to have antioxidant, hypolipidemic, anti-atherosclerotic, central nervous system stimulant, and anti-fertility properties (8). The dried root of *Plumbago zeylanica* holds significant value in Ayurvedic medicine for treating various ailments such as rheumatic fever, dyspepsia, piles, diarrhea, skin diseases, leprosy, leukoderma, paralysis, epilepsy, and hysteria. Various parts of Plumbago zeylanica contain several phytochemical constituents including plumbagin, dihydroserone, elliptinone, droserone, isoshinanolone, Plumbazeylanone, Zeylanone, 3 – Chloroplumbagin, Isozeylanone, and 8,8'-biplumbagin. Among these, plumbagin has demonstrated anti-diabetic effects in streptozotocin induced diabetes in rats by promoting glucose homeostasis (9). Plumbagin has been found to possess inhibitory effects on T cell proliferation and the activation of NF kappaβ, thereby exhibiting immunomodulatory properties in mice (10).

The present study aimed to compare and evaluate the nephroprotective potential of hydroalcoholic extracts derived from different parts of *Plumbago zeylanica*, namely the root, stem, and leaf. *In-vitro* experiments were conducted to investigate the diabetic nephropathy properties of *Plumbago zeylanica*. Additionally, to assess the binding affinity of the phytocompounds present in *Plumbago zeylanica* with target proteins associated with diabetes and diabetic nephropathy, *in-silico* molecular docking was performed against specific target proteins associated. To predict the ADME-Toxicity of the phytocompounds present in *Plumbago zeylanica*, a web server like pkCSM was used.

2. Materials and Methods

2.1. Materials

Soxhlet apparatus, round bottom flask, China dish, desiccators reflux condenser, measuring cylinder, test tubes, grinding mixer, hot air oven, UV-Visible spectrometer (Perkin Elmer Lambda 25 Uv-vis Spectrometer), Ethanol (Rankem), Distilled water, Rutin standard. α-amylase and α-glucosidase (Sigma-Aldrich, Inc., Darmstadt, Germany), Sulphuric acid, HCl, methanol and ethanol (LR grade and AR grade) purchased from Merck, Mumbai, India.

2.2. Plant authentication and Extraction

The fresh plant parts (root, stem, and leaf) of *Plumbago zeylanica* were procured from Global Herbs, Khari Baoli New Delhi. Before carrying out the research work, the collected plant materials were authenticated by an Emeritus Scientist at NISCAIR with matching the authentic samples in the Raw material herbarium and Museum, Delhi. The authentication reference number of root, stem and leaf are NIScPR/RHMD/Consult/2022/4218-19, NIScPR/RHMD/Consult/2023/4371-72 and NIScPR/RHMD/ Consult/2022/4437-38.

The plant parts (root, stem, and leaf) of *Plumbago zeylanica* were separated, thoroughly cleaned to remove adhering dust and other foreign materials then washed with water. The stem, leaf and root were air-dried, powdered with the help of a grinder (USHA Lexus 2753), and kept in an airtight container until use. The coarse powder of dried plant parts (root, stem, and leaf) was extracted with hydroalcoholic solvent (Ethanol: water) in ratio of 50:50 by Soxhlet extraction for 72 h approx. The extracts were concentrated under reduced pressure at 45 °C in a vacuum rotary evaporator and lyophilized to give a dry extract. The extracts obtained were weighed and percentage yield was recorded.

2.3. Phytochemical Estimation of Plant extracts

2.3.1. Total Phenolic Contents estimation

The TPC of the samples was assessed using the Folin–Ciocalteau's assay based on the (11) method with some modifications. For extracts (1mg/ml), 1mL of sample containing 1.0mg/mL of a standard gallic acid solution was mixed with 0.5mL of the Folin-Ciocalteau's reagent and left at room temperature for 3 minutes. The Na2CO3 solution was prepared at a concentration of 7.5% (w/v),

heated for 1 minute at 95ºC, and cooled to room temperature. The sample or gallic acid solution was then treated with 2.0mL of 7.5% Na2CO3. The absorbance of the sample was measured at 760 nm using a UV-vis spectrometer after 1 hour of incubation at room temperature in the dark. The calibration curve was plotted using gallic acid (mg) as a standard.

2.3.2. Total Flavonoid Contents estimation

The total flavonoid content (TFC) of each extract was investigated using the aluminum chloride colorimetry method described by (12) with slight modifications. To prepare the sample, ethanol was used to dilute the extract until it reached a concentration of 100µg/mL. For the calibration curve, a quercetin solution (1mg/ml) was prepared. Next, a mixture was prepared by combining 2.0mL of the diluted extract or quercetin with 0.1mL of a 10% (w/v) solution of aluminum chloride and 0.1mL of a 0.1mM potassium acetate solution. The mixture was allowed to stand at room temperature for 30 minutes. Subsequently, the UV-vis spectrophotometer was employed to measure the absorbance of the mixture at 415 nm. The total flavonoid content (TFC) was determined by expressing it as milligram quercetin equivalent per gram (mg QE/g).

2.3.3. Antioxidant study

The antioxidant activity of the hydroalcoholic extracts of *Plumbago zeylanica* was determined by evaluating their ability to scavenge the stable 2, 2-diphenyl-2-picrylhydrazyl (DPPH) free radical (13). The following concentrations of extracts ranging from 20-150µg/ml was prepared. Each prepared concentration was mixed with 0.5 ml of 1 mM DPPH solution in methanol. The test tubes were incubated at room temperature for 30 minutes, and the absorbance was measured at 517 nm. A lower absorbance of the reaction mixture indicates a higher free radical scavenging activity. Ascorbic acid was used as a standard, and solutions with the same concentrations were prepared as the test solutions. The difference in absorbance between the test and the control (DPPH in ethanol) was calculated and expressed as the percentage scavenging of the DPPH radical. The experiment was conducted in triplicate. The capability to scavenge the DPPH radical was determined using the following equation:

Scavenging effect (%) =(I-As/Ac) x 100
As is the absorbance of the sample at t =0 min.
Ac is the absorbance of the control at t=30 min.

2.4. *In-Silico* Experiments

Molecular modeling studies were performed for the identified bioactive compounds from the *Plumbago zeylanica* extract to assess the ability for inhibitory activity against target protein associated with diabetic nephropathy.

2.4.1. Ligand Preparation and Drug-Likeness Analysis

A total of 11 phytocompounds of *Plumbago zeylanica* were selected for the docking analysis, including 3-chloro plumbagin, Chitranone, Plumbagin, Zeylanone, Isozeylanone, Elliptinone, Methylnaphthazarin, Plumbagic acid, and 8,8'-Biplumbagin. The plants phytocompounds were taken from "The Indian Medicinal Plants, Phytochemistry, and Therapeutics" (IMPPAT) database (website: https:// cb.imsc. res.in/imppat/). Ligands were downloaded from PubChem (website: https:// pubchem.ncbi.nlm.nih.gov/).
The reference compounds used for this study were lisinopril as ACE inhibitor (14), epalrestat as ALR inhibitor(15), sitagliptin as DPP-4 inhibitor (16), tesaglitazar as PPAR- γ & PPAR-α agonist (17), aminoguanidine as RAGE inhibitor (18), the standard treatment for diabetic nephropathy (19).
The ligand molecules used in this study were obtained from PubChem (https://pubchem. ncbi.nlm.nih.gov) in the structure data format (.sdf). To convert the ligand molecules into their 3D structure, we utilized Open Babel version 2.4.1 software.
To qualify the selected phytocompounds in *Plumbago zeylanica* as drug candidates, the properties of Lipinski's rule of five were calculated using Swiss ADME website (http://www.swissadme.ch/) (20). Only ligand molecules that adhered to Lipinski's rule were selected for docking in the study.

2.4.2. Retrieval of protein targets

The 3D structures of diabetes-related proteins were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org) with the following identifiers: ACE (PDB ID 4APH), ALR (PDB ID 1US0), DPP-4 (PDB ID 5Y7H), PPAR-γ (PDB ID 4EMA), PPAR-α (PDB ID 2P54), and RAGE (PDB ID 4LP4).

2.4.3. Molecular Docking Studies

Schrödinger maestro version 13.1., 2022-01 release was used to perform molecular docking for predicting free binding energy. All 11 phytocompounds of *Plumbago zeylanica* that passed the drug-likeness criteria then were docked

with nine protein targets, including ACE (lisinopril as reference compound), ALR (epalrestat as reference compound), DPP-4 (sitagliptin as reference compound), PPAR-α (tesaglitazar as reference compound), PPAR-γ (tesaglitazar as reference compound), and RAGE (aminoguanidine as reference compound). Binding interaction and the residues of proteins involved in the interaction with the ligands were analysed using Protein-Ligand Interaction Profiler (PLIP) web server tool (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index).

2.4.4. ADME-T Prediction

The absorption, distribution, metabolism, elimination, and toxicity (ADMET) properties of the 11 phytocompounds of *Plumbago zeylanica* were evaluated using three distinct web servers to predict their significant pharmacokinetic properties. One of the web servers employed for this analysis was pkCSM, accessible at the following URL: https://biosig.unimelb.edu.au/pkcsm (21,22). The ADMET properties encompass various aspects of a compound's behaviour in the human body. These properties include:

1.	Absorption	Human Intestinal Absorption (HIA) Human Oral Bioavailability (HOB) CaCo-2 Permeability Water Solubility (logS) Subcellular Localization
2.	Distribution	P-glycoprotein (P-gp) Substrate and Inhibitor Blood-Brain Barrier (BBB) Permeability
3.	Metabolism	CYP450 (2C9, 2D6, 3A4) Substrate CYP450 (1A2, 2C9, 2D6, 2C19, 3A4) Inhibitor
4.	Excretion	Renal Organic Cation Transporter-2 (rOCT-2) Substrate and Inhibitor
5.	Toxicity	AMES Toxicity Carcinogens Hepatotoxicity

These properties help in evaluating the behaviour, efficacy, and safety of compounds in pharmacokinetics and toxicological studies.

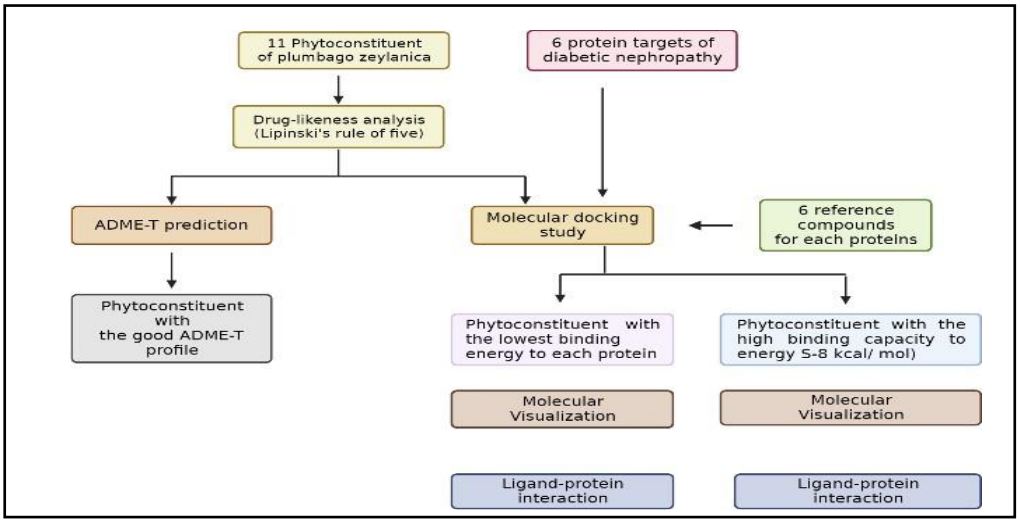


Figure 1. Flowchart showing the steps analysis of this *in-silico* study

2.4.5. Data Analysis

Figure 3 provides a summary of the data analysis steps involved in the *in-silico* experiments. The primary objectives of the data analysis were as follows:

- To propose the molecular anti-diabetic and nephroprotective mechanism of *Plumbago zeylanica* by identifying the phytocompounds that exhibits the lowest energy binding to each target protein.
- To identify the phytocompounds present in *Plumbago zeylanica* that have a high binding capacity (≤-8 kcal/mol) to various proteins associated with diabetic nephropathy.
- To predict the phytocompounds in *Plumbago zeylanica* that possess favourable ADME-T

properties, including absorption, distribution, metabolism, elimination, and toxicity.

These analyses were conducted to gain insights into the potential therapeutic effects of *Plumbago zeylanica* phytocompounds for diabetic nephropathy treatment and to assess their pharmacokinetic properties and safety profiles.

2.5. *In-Vitro* Experiments

2.5.1. α- Amylase Inhibition Study

The modified standard method was used to determine the α-amylase inhibitory activity (23). 20 mM phosphate buffer pH 6.9 with α-amylase at a concentration of 0.5mg/mL and 25µl of 20% (v/v) *Plumbago zeylanica* extract were incubated at 25°C for 10 min. 25µl of a 0.5% starch solution in a 20 mM phosphate buffer with a pH 6.9 were added after pre-incubation. Following that, the reaction mixtures were incubated for 10 minutes at 25°C. With 50µl of 96 mM 3, 5-dinitro salicylic acid (DNS), the reaction was stopped.

The microplate was then cooled at room temperature after 5 minutes of incubation in a boiling water bath. At 540 nm, absorbance (A) was measured. The % of α-amylase inhibitory activity was calculated by the following equation:

% α- Amylase Inhibitory Activity: Ac- (At - Ab) /Ac X 100

Ab background is defined as the absorbance of 100% enzyme activity, the test sample with the enzyme, and the test sample without the enzyme, respectively, where Ac is control and At is test. The IC50 values represent the inhibitor concentrations necessary to inhibit 50% of the enzyme activity during the assay.

2.5.2. α- Glucosidase Inhibition Study

Briefly, 96 well plates were incubated at 37 ºC for 20 min with 60µl of the *Plumbago zeylanica* extract in DMSO or acarbose in DMSO and 50µl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2U/mL). Each well underwent a 20-minute pre-incubation period before receiving 50 µl of a 5 mM para-nitrophenyl-D-glucopyranoside (PNPG) solution in 0.1M phosphate buffer (pH 6.8). After that, the reaction was stopped by pouring 160µl of 0.2M Na2CO3 into each well. An absorbance reading at 405 nm was then taken, and the results were compared to a control that contained 60 µl of buffer solution instead of the *Plumbago zeylanica* extract of the relevant plants. After replacing the enzyme solution with buffer solution for the blank incubation period, absorbance was measured. The following equation was used to calculate the percent inhibition of α-glucosidase inhibitory activity:

Glucosidase Inhibitory Activity — Ac- (At - Ab) /Ac X 100

Where Ac stands for "control," At for "test," and Ab for "background," respectively, those terms refer to the absorbance of 100% enzyme activity, test samples containing the enzyme, and test samples lacking the enzyme. The IC50 values represent the inhibitor concentrations necessary to inhibit 50% of the enzyme activity during the assay.

2.5.3. Glucose Uptake Assay in Yeast

Saccharomyces cerevisiae, the common name for baker's yeast, was grown in yeast malt medium, and the culture was centrifuged for five minutes at a speed of 3000 g. For the experiment, yeast cell pellets were taken out and the supernatant was discarded. In addition, sterilized deionized water produced a 10% (v/v) yeast suspension. To 1.0 ml of glucose solution (5, 10 and 25mM), different concentrations of *Plumbago zeylanica* extract (3 to 5mg/ml) were added. The mixture was then incubated both separately and simultaneously for 10 min at 37 °C. Each extracted glucose solution received 100µl of yeast suspension to begin the glucose uptake process. After thoroughly mixing the mixture, it was incubated at 37 °C for 30 min. The suspension was centrifuged for 5 minutes at 3000 g after 1 hour, and the supernatant was pipetted out to determine the presence and amount of glucose (24). The formula below was used to calculate the percentage increase in glucose uptake in yeast cells caused by *Plumbago zeylanica* extracts:

Increase in glucose uptake = (absorption control - absorption sample)

Absorption control x 100

Where, the absorbance of the sample reaction containing extracts of *Plumbago zeylanica* and the absorbance of the control reaction containing all reagents but not the test sample.

3. Results

3.1. Extraction

The coarse powder of dried plant parts (root, stem, and leaf) of *Plumbago zeylanica* were extracted with hydroalcoholic (water: ethanol) in solvent ratio of Soxhlet extraction for 72 h each (25-28). It was observed that the highest yield of extract is of root, followed by stem and then leaf.

Table 1. Extractive value of hydroalcoholic extracts of *Plumbago zeylanica*

Plant parts	Content of extracts
Root	13.7%
Stem	7.02%
Leaf	2.43%

3.2. Total phenolic estimation

The total phenolics of *Plumbago zeylanica* plant parts (root, stem, and leaf) were investigated. The total phenolics value was found to be 185.88 ± 7.51 mg/g of gallic acid equivalent (GAE)/g (D.W.) in root, 154.28 ± 3.53 mg/g of gallic acid equivalent (GAE)/g (D.W.) in the stem, and in the leaf, value was found to be 131.16 ±3.19 mg/g of gallic acid equivalent (GAE)/g (D.W.) (29-32)

3.3. Total flavonoid estimation

The flavonoid content of *Plumbago zeylanica* plant parts extract (root, stem, and leaf) were investigated. The total flavonoid value was found to be 180.65 ± 3.84 mg/g of quercetin equivalent (QE)/g (D.W.) in root of *Plumbago zeylanica*. In the case of *Plumbago zeylanica* stem., total flavonoid value was found to be 120.1 ±4.47 mg/g of quercetin equivalent (QE)/g (D.W.). Whereas, in the case of P*lumbago zeylanica* leaf, the total flavonoid value was found to be 107.1 ± 2.76 mg/g of quercetin equivalent (QE)/g (D.W.). (33-35)

Table 2. Total phenolic content of *Plumbago zeylanica* extracts

Plant part extracts	Total phenolics content (% w/w)	Total Flavonoid content (% w/w)
Root	185.88 ± 7.51	180.65 ± 3.84
Stem	154.28 ± 3.53	120.1 ±4.47
Leaf	131.16 ±3.19	107.1 ± 2.76

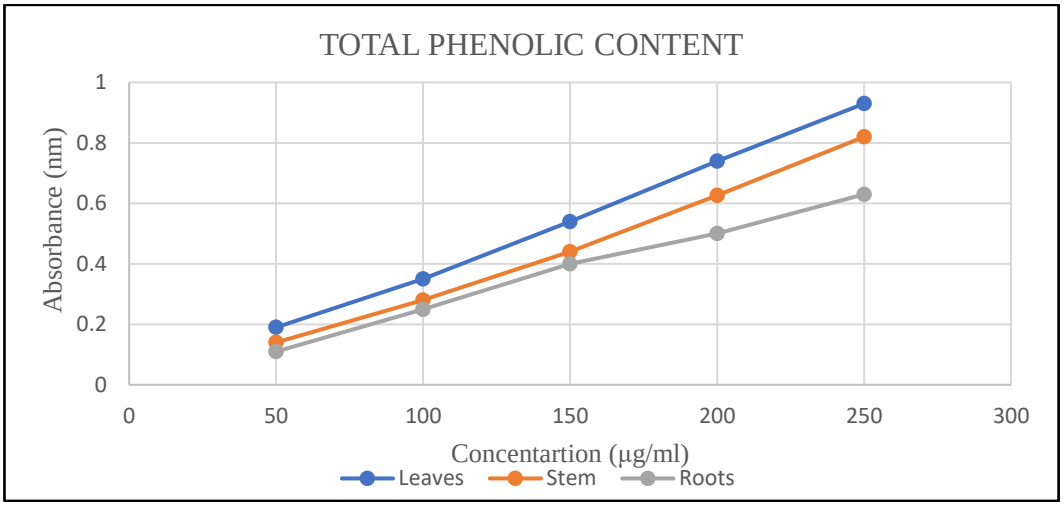


Figure 2. Calibration curve of Total Phenolic Content

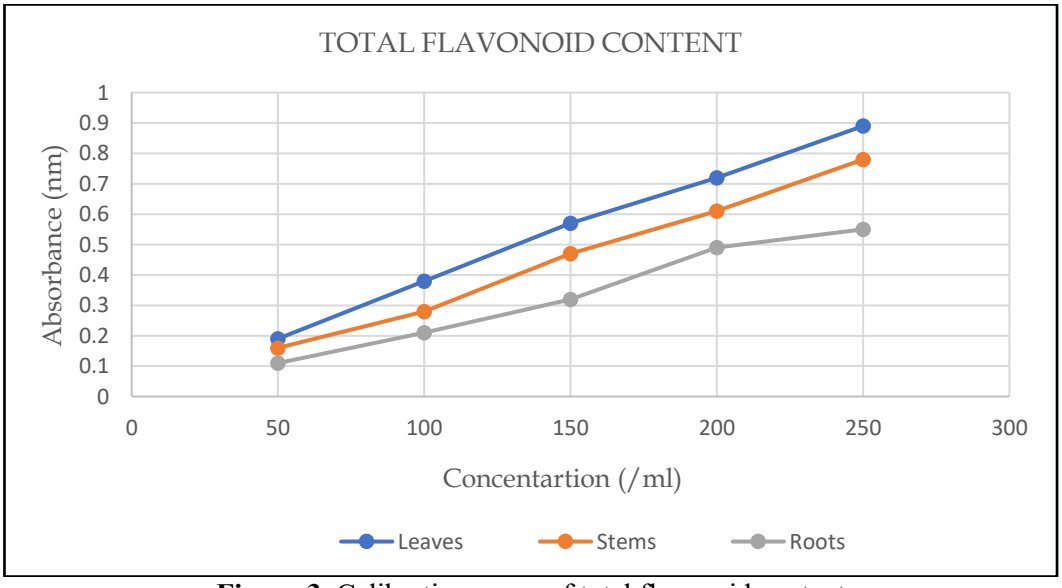


Figure 3. Calibration curve of total flavonoid content

3.4. Antioxidant Study By 1, 1-Diphenyl-2 Picrylhydrazyl Radical (DPPH) Method

Hydroalcoholic extract of *Plumbago zeylanica* root exhibited a significant ($p < 0.001$) dose dependent inhibition of DPPH activity with 20.93, 31.86, 46.65, 65.99, 72.68, 74.95, 76.12 % inhibition at 20, 40, 60, 80, 100, 125 and 150 µg/mL concentrations respectively. Here, the 50% inhibition (IC) was obtained at a concentration of 60 µg/mL. Whereas extract of *Plumbago zeylanica* stem exhibited a significant ($p < 0.001$) dose dependent inhibition of DPPH activity with 21.42, 29.4, 39.22, 52.73, 59.48, 65.009, 61.32 % inhibition at 20, 40, 60, 80, 100, 125 and 150 µg/mL concentrations respectively. Here, the 50% inhibition (IC) was obtained at a concentration of 80 µg/mL. At last, extract of *Plumbago zeylanica* leaf exhibited a significant ($p < 0.001$) dose dependent inhibition of DPPH activity 20.81, 28.79, 31.24, 35.54, 38.61, 43.52, 48.43% inhibition at 20, 40, 60, 80, 100, 125 and 150 µg/mL concentrations respectively. Here, the 50% inhibition (IC) was obtained at a concentration 150 µg/mL. The ascorbic acid exhibits a significant ($p < 0.001$) dose dependent inhibition of DPPH activity 19.89, 31.83, 50.95, 70.73, 89.16, 90.89, 92.57 % inhibition at 20, 40, 60, 80, 100, 125 and 150 µg/mL concentrations respectively. Here, the 50% inhibition (IC) was obtained at a concentration of 50 µg/mL.

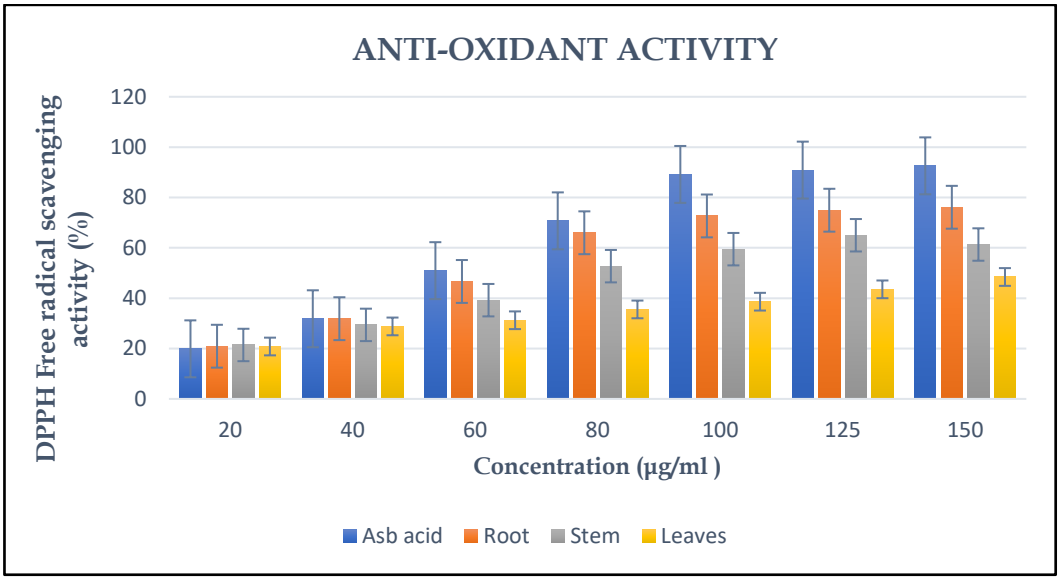


Figure 4. Graphical representation of antioxidant activity of Standard (Ascorbic acid) and *Plumbago zeylanica* extracts

3.5. *In-vitro* Experiments

3.5.1. Inhibition Activity Of α- Amylase And α-Glucosidase

The digestive enzymes (α-amylase and α-glucosidase) inhibitors are effective for the treatment of diabetes, obesity, and hyperlipidemia. Medicinal plants involve retarding the absorption of glucose by inhibiting the carbohydrate hydrolyzing enzymes. The inhibition of α- amylase by the *Plumbago zeylanica* extracts (Root, stem, and leaf) were performed at different concentrations (50-500µg/mL). The inhibitory potentials of hydroalcoholic extracts of *Plumbago zeylanica* was found to be significant with an IC50 value of 45.28, 41.57, 38.27 mg/mL. α-glucosidase inhibitory potential of *Plumbago zeylanica* extracts (Root, stem, and leaf) was also determined. The prepared extract was tested for α-glucosidase inhibitory potential at (50-500 µg/ml). The results revealed that the extract is active in inhibiting α-glucosidase with an IC50 value of 45.76, 44.55, and 42.47 mg/mL.

Table 3. α-amylase and α-glucosidase inhibition

Plant Extract	α-amylase inhibition (IC_{50})	α- glucosidase inhibition (IC_{50})
Root	38.27	42.47
Stem	41.57	44.55
Leaf	45.28	45.76
Acarbose	35.81	40.69

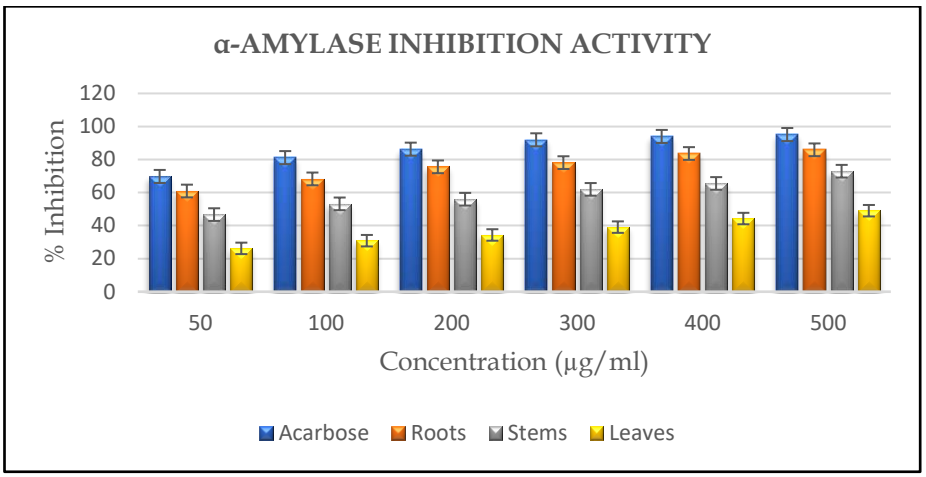


Figure 5. Graphical representations of α-amylase inhibition potential of *Plumbago zeylanica*

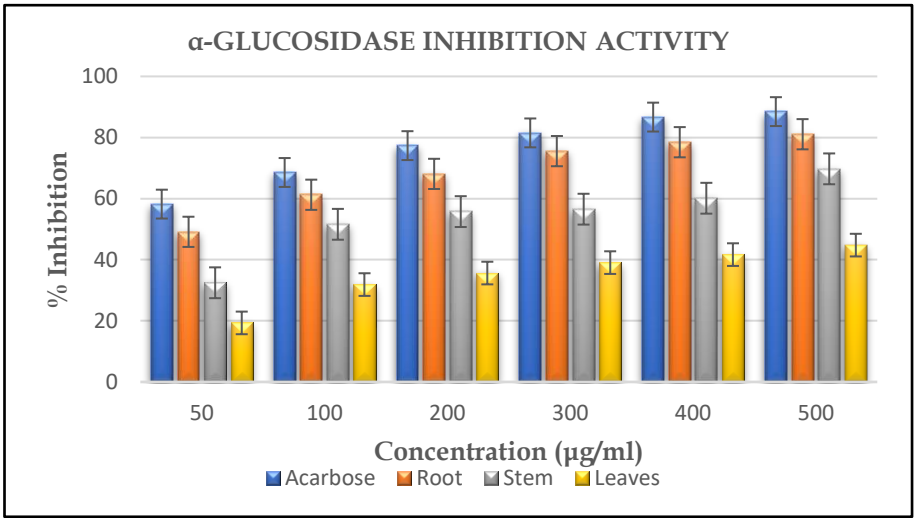


Figure 6. Graphical representations of α-glucosidase inhibition potential of *Plumbago zeylanica*

3.5.2. Glucose Uptake Assay In Yeast Cell

The hydroalcoholic extracts of *Plumbago zeylanica* were further tested for its potential to inhibit glucose uptake in yeast. Transportation of glucose in the baker's yeast cell was done by a route i.e., facilitated diffusion method. After the treatment of the *Saccharomyces* yeast cell with the hydroalcoholic extracts of *Plumbago zeylanica*, results obtained and predicted to be as no dose dependent. The percentage of glucose uptake by the yeast cell directly depends on the glucose concentrations in the medium. In the presence of hydroalcoholic extract of *Plumbago zeylanica* root, that 93.56 % glucose uptake was found in the presence of 10 mM glucose. Figure 7, 8 and 9 demonstrated the relationship between glucose concentrations and extracts condition i.e., 50, 100, and 150mM.

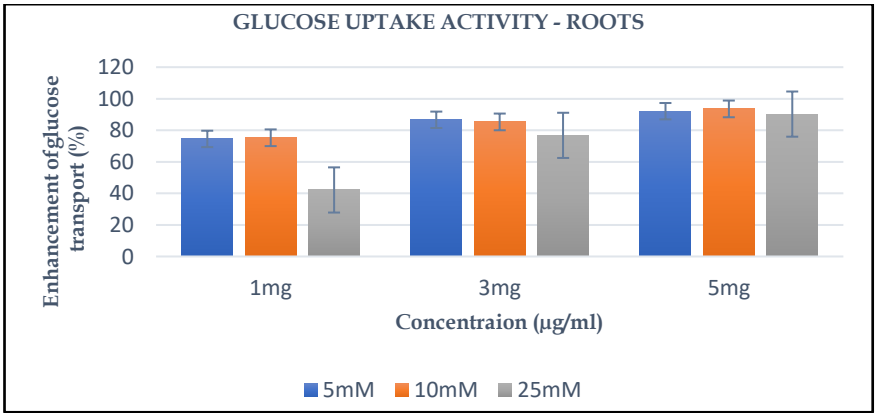


Figure 7. Graphical representation of Glucose uptake activity of *Plumbago zeylanica* root extract

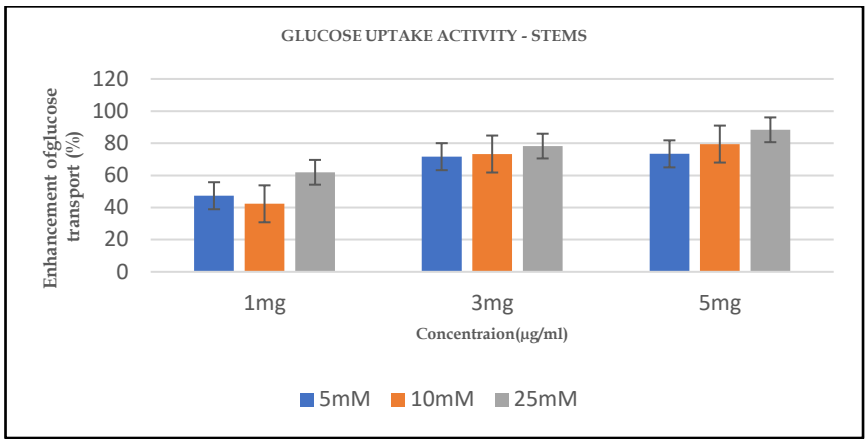


Figure 8. Graphical representation of Glucose uptake activity of *Plumbago zeylanica* stem extract

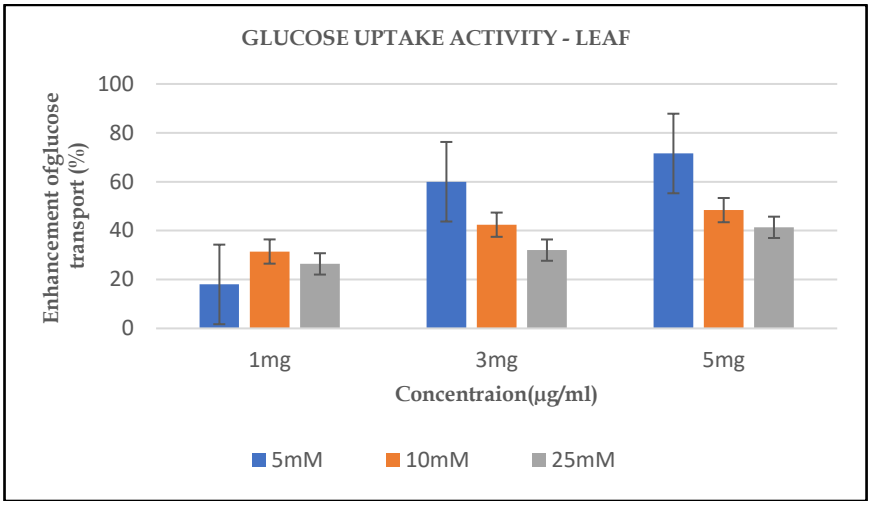


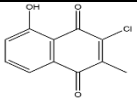
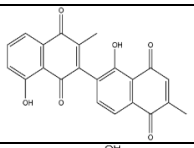
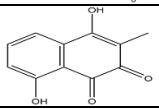
Figure 9. Graphical representation of Glucose uptake activity of *Plumbago zeylanica* leaf extracts

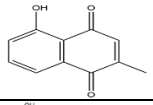
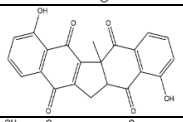
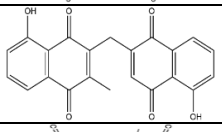
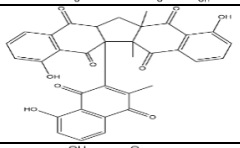
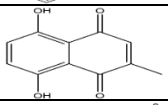
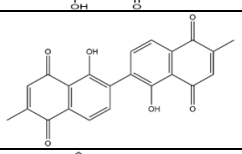
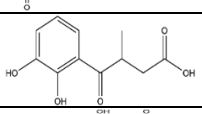
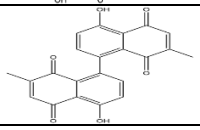
3.6. *In-Silico* Experiments

3.6.1. Drug Likeness Analysis of Phytocompounds in *Plumbago Zeylanica*

Lipinski's rule of five is used to analyze whether a molecule has the potential to be developed as a drug (drug- likeness). This rule predicts that the molecule has a high probability to developed as a drug if there are no more than two rule violations on the following criteria: there are no more than five H-bond donors (HBD) and 10 H- bond acceptors (HBA), the molecular weight (MWT) is smaller than 500, and the calculated Log P (CLogP) is smaller than 5 (Lipinski et al., 2012; 44). Based on the drug analysis results presented in Table 4, the molecular weight, the number of hydrogen bond acceptors and donors, and the lipophilicity property of all the phytocompounds in *Plumbago zeylanica* were evaluated according to Lipinski's rule of five. The compounds that meet Lipinski's criteria were selected for the molecular docking study.

Table 4. Lipinski's rule of five for the phytocompounds in *Plumbago zeylanica*

S.no.	Phytocompounds	Structures	Mol. Wt.	Log P	HBD	HBA	No. of rule violations	Drug Likeness
1.	3-chloro plumbagin		222.63	2.28	1	3	0	Yes
2.	Chitranone		374.35	3.28	2	6	0	Yes
3.	Droserone		204.18	1.45	2	4	0	Yes

4.	Plumbagin		188.18	1.72	1	3	0	Yes
5.	Zeylanone		374.35	2.88	2	6	0	Yes
6.	Isozeylanone		374.35	3.19	2	6	0	Yes
7.	Plumbazeylanone		476.56	4.68	3	9	0	Yes
8.	Elliptinone		374.35	3.42	2	6	0	Yes
9.	Methylnaphthazarin		204.18	1.42	2	4	0	Yes
10.	Plumbagic acid		224.21	1.39	3	4	0	Yes
11.	8,8'-Biplumbagin		374.35	3.8	2	6	0	Yes

3.6.2. Molecular Docking Study

Molecular docking was conducted to assess the potential anti-diabetic effect of *Plumbago zeylanica* phytocompounds on multiple targets related to diabetic nephropathy. The docked binding energy of the selected ligands against each protein is presented in Table 5. The binding free energies of the compounds ranged from -5.1 to -9.5 kcal/mol. These binding energies provide insights into the strength of interaction between the phytocompounds and their respective target proteins, suggesting their potential as anti-diabetic nephropathy agents (36-38). In this study, we revealed the compounds with the highest binding affinity for PPAR-α was Plumbagin, Elliptinone, 8,8'-Biplumbagin, Plumbagic acid.; for ALR and DPP-4 were 8,8'- Biplumbagin, Plumbagic acid; for PPAR-γ were Elliptinone; for RAGE was Chitranone, Droserone, Zeylanone, Elliptinone, 8,8'-Biplumbagin, Plumbagic acid.

Table 5. Summary of molecular docking result

PHYTOCOMPOUNDS/ PROTEINS	5Y7H	1US0	4EMA	2P54	4APH	4LP4
Reference	-7.432	-8.631	-4.713	-6.614	-12.529	-2.686
A1: 3-chloro plumbagin	-5.721	-6.935	-3.691	-4.165	-2.463	-2.036
A2: Chitranone	-	-6.362	-2.8	-5.684	-5.151	-4.274
A3: Droserone	-5.355	-5.227	-3.13	-2.153	-2.882	-3.981
A4: Plumbagin	-6.147	-6.795	-2.933	-8.367	-4.172	-2.172
A5: Zeylanone	-	-6.655	-3.075	-	-4.35	-4.397
A6: Isozeylanone	-	-8.608	-3.645	-5.099	-4.654	-3.01
A7: Elliptinone	-	-7.901	-5.412	-7.467	-2.835	-4.352
A8: 8,8'-Biplumbagin	-	-9.196	-3.357	-8.118	-4.317	-3.929
A9: Methylnaphthazarin	-7.465	-6.187	-1.781	-6.635	-4.381	-
A10: Plumbagic acid	-8.369	-9.089	-2.588	-7.196	-5.049	-3.779

The molecular interactions of the amino acid residues of RAGE, ALR, PPAR-α and DPP-4 with the highest binding affinity compounds against each protein are presented in table 6. 3-chloro plumbagin, Plumbagin, Isozeylanone, Methylnaphthazarin achieved the lowest free energy binding than aminoguanide, a RAGE inhibitor. Plumbagin, Elliptinone, 8-8' biplumbagin and Plumbagic acid showed higher binding affinity than reference compound for PPAR-α. 8-8' biplumbagin and plumbagic acid showed higher affinity than reference compound for Aldose reductase. Only Plumbagic acid showed higher Binding affinity than reference compound for DPP-4. All 11 phytocompounds exhibited a lower free binding energy to ACE than lisinopril (-12.529Kcal/mol). Most constituents of *Plumbago zeylanica* showed a lower free binding energy to ALR, 4EMA, 4APH and 2P54. The free binding energy to targets were lowest for 3-chloro plumbagin, Droserone, Methylnaphthazarin and largest for Plumbagin, Elliptinone, 8,8'-Biplumbagin, Plumbagic acid.

Table 6. binding interactions of protein targets with their ligands

PDB ID	Ligands	Docking score	Amino acid residues	Type of bonds
1US0	8,8'-Biplumbagin	-9.196	TRP20, HIS110, TRP111 TRP20	H-BOND Pi-Pi stacking
1US0	Plumbagic acid	-9.089	THR19, TRP20, LYS21, ASP43, LEU212, SER214, LYS262 TYR209, ILE260	H-BOND H-BOND Hydrophobic
2P54	Bi-plumbagin	-8.118	ALA333, ILE241, ALA250, LEU254, VAL255, THR279, VAL332, TYR334	H-BOND Hydrophobic Hydrophobic
2P54	Plumbagin	-8.367	HIS440, TYR314, TYR464 PHE273, TYR314, PHE318, LEU456, LEU460, TYR464	H-BOND Hydrophobic Hydrophobic
2P54	Elliptinone	-7.467	THR279, ALA333, TYR334 ILE241, GLU251, VAL255, THR279, VAL332, ALA333, TYR334	H-BOND Hydrophobic Hydrophobic Hydrophobic
2P54	Plumbagic acid	-7.196	SER280, TYR314, TYR464 PHE273, LEU321, ILE354	H-BOND Hydrophobic
5Y7H	Plumbagic acid	-8.369	SER239, TYR241, THR706, PHE713, GLN714 TYR238, TYR241, THR706, PHE 713	H-BOND H-BOND Hydrophobic
4LP4	Zeylanone	-4.397	GLU32, LYS37 PRO33, VAL35	H-BOND Hydrophobic
4LP4	Elliptinone	-4.352	VAL35, LYS37 GLU32, LEU34, LYS37, TYR113	H-BOND Hydrophobic
4LP4	Droserone	-3.981	VAL35, LYS37 VAL35	H-BOND Hydrophobic
4LP4	8,8'-Biplumbagin	-3.929	GLU32, PRO33, VAL35 LEU34, VAL35	H-BOND Hydrophobic
4LP4	Plumbagic acid	-3.779	THR27, ARG29, PRO33, VAL35 PRO33, LEU34, VAL35	H-BOND Hydrophobic
4EMA	Elliptinone	-5.412	ARG397, TYR320, VAL446, VAL450	H-BOND Hydrophobic

The docked complexes with the highest binding affinity are visualized in Figures 10-22 , and their amino acid residues is shown in table 6.

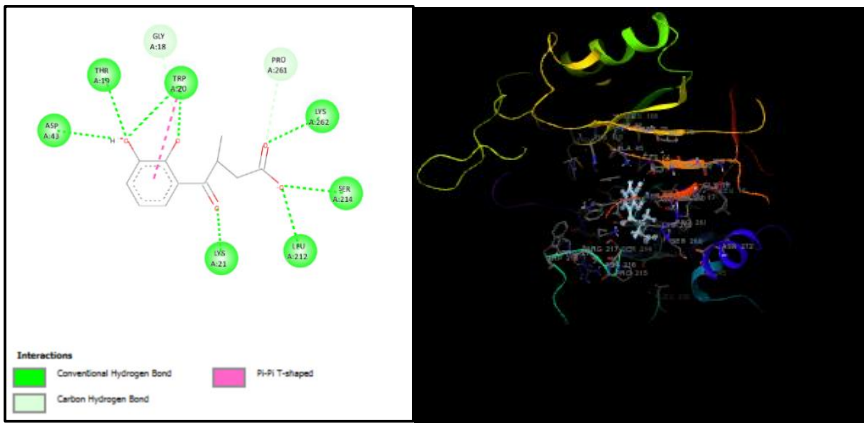


Figure 10. 2D and 3D interaction of 1US0 & plumbagic acid

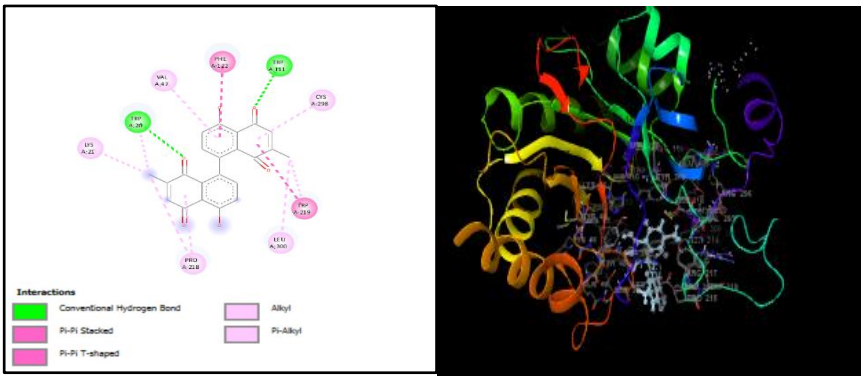


Figure 11. 2D and 3D interaction of 1US0 & biplumbagin

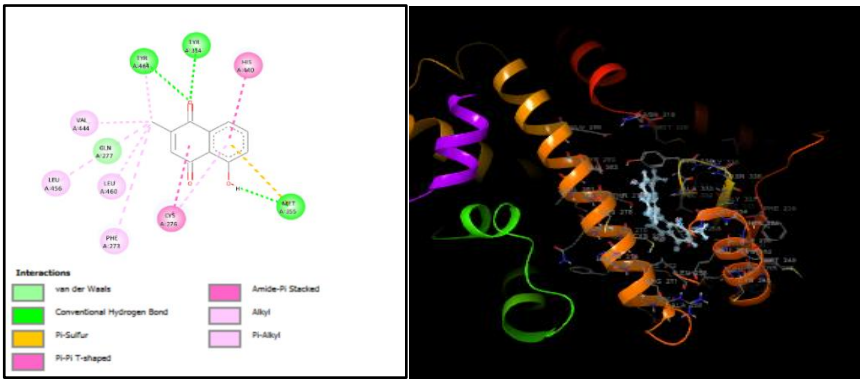


Figure 12. 2D and 3D interaction of 2P54 & plumbagin

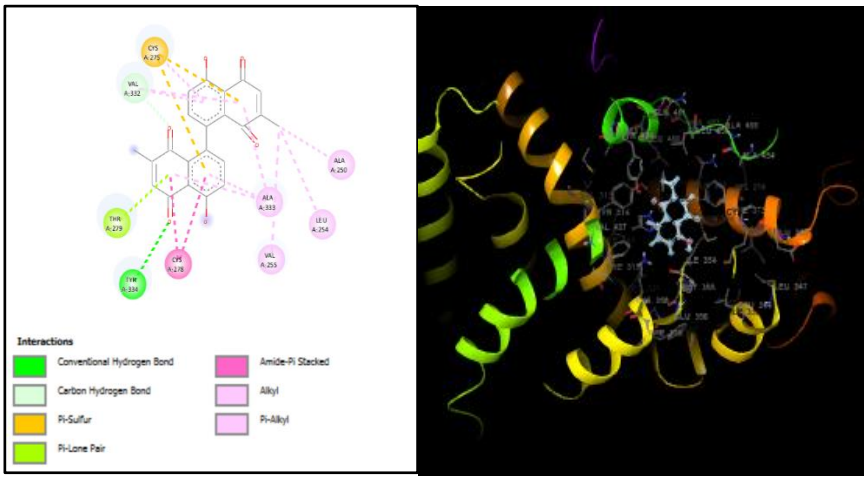


Figure 13. 2D and 3D interaction of 2P54 & biplumbagin

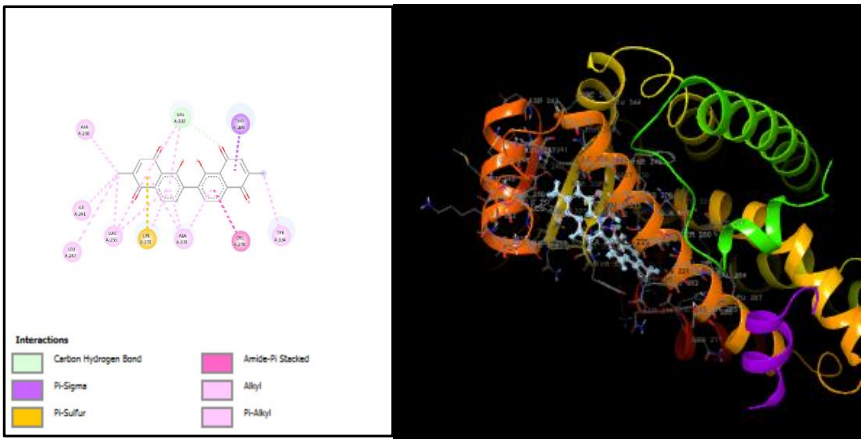


Figure 14. 2D and 3D interaction of 2P54 & elliptinone

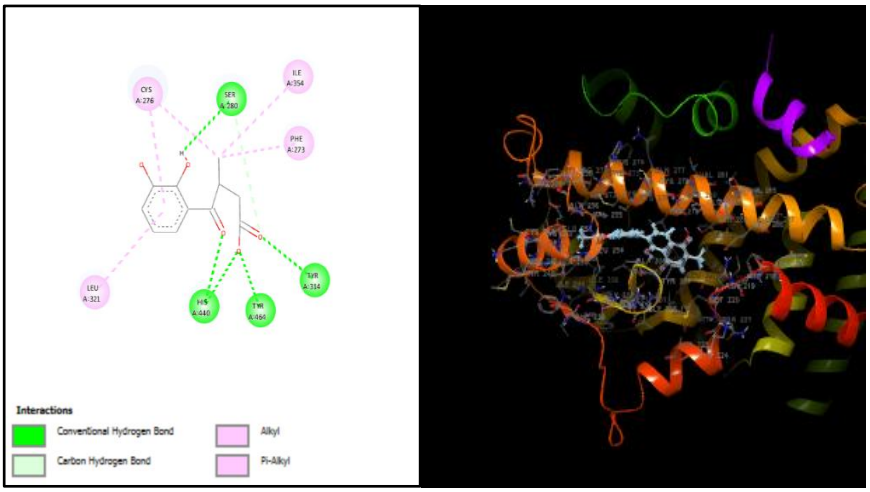


Figure 15. 2D and 3D interaction of 2P54 & plumbagic acid

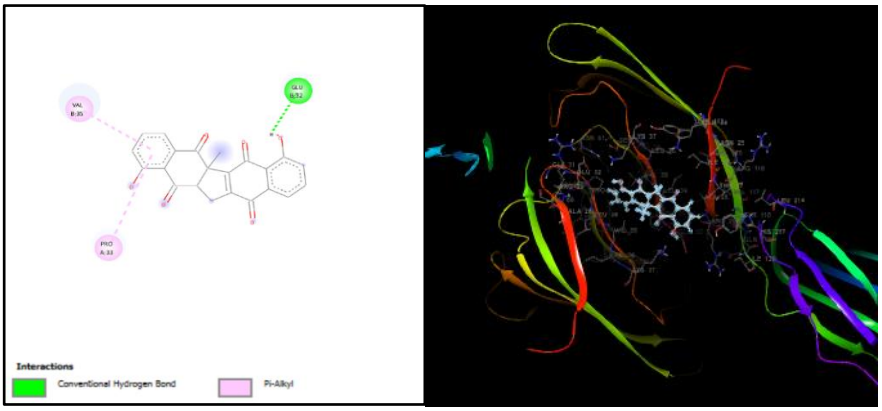


Figure 16. 2D and 3D interaction of 4LP4 & Zeylanone

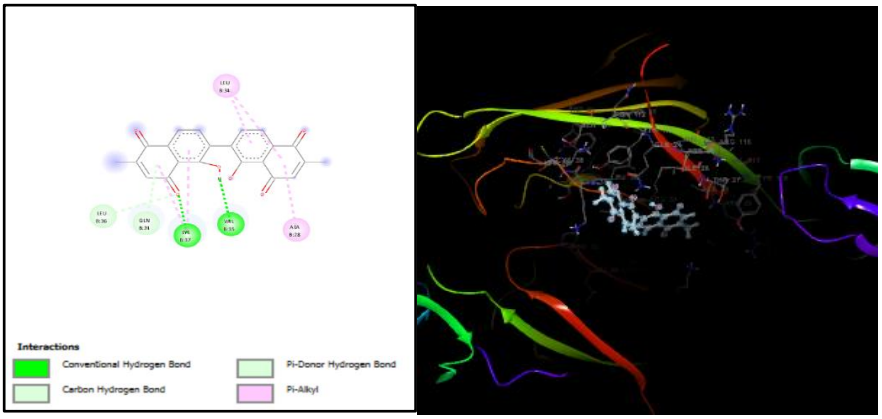


Figure 17. 2D and 3D interaction of 4LP4 & Elliptinone

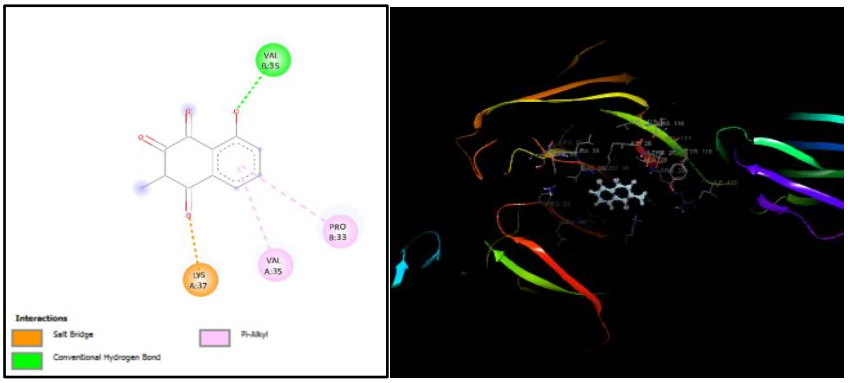


Figure 18. 2D and 3D interaction of 4LP4 & Droserone

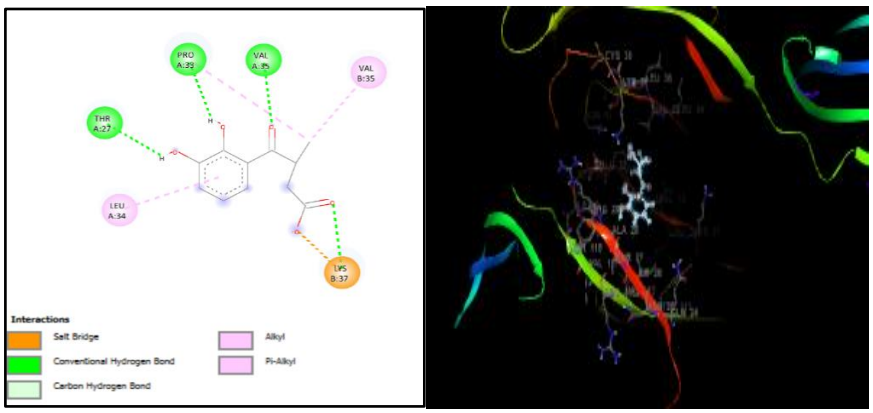


Figure 19. 2D and 3D interaction of 4LP4 & plumbagic acid

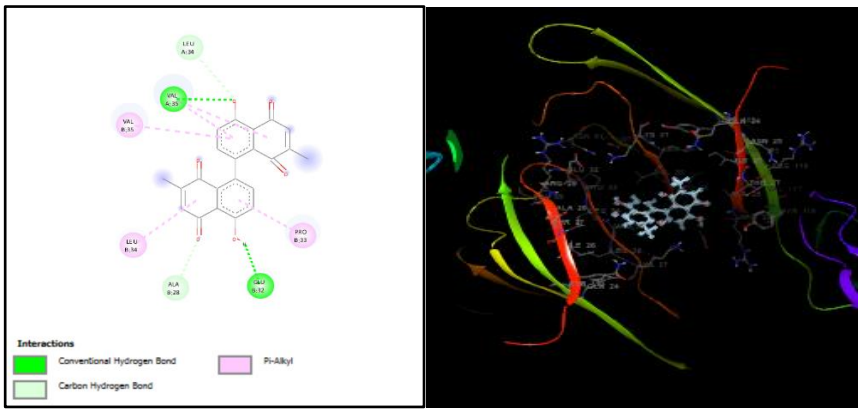


Figure 20. 2D and 3D interaction of 4LP4 & biplumbagin

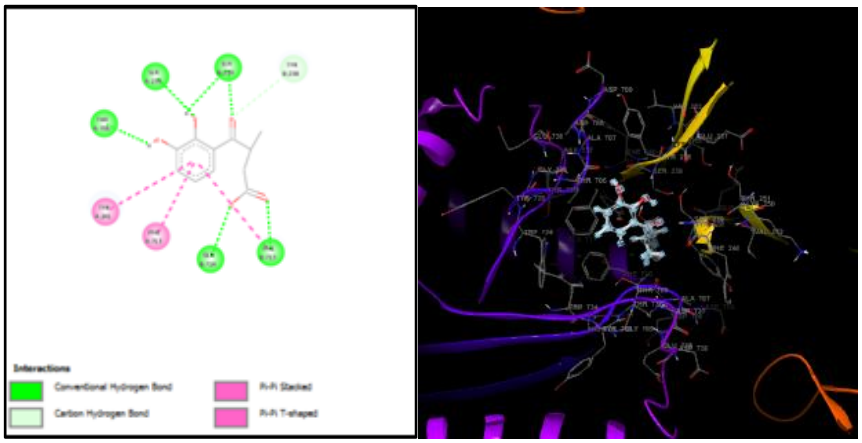


Figure 21. 2D and 3D interaction of 5Y7H & plumbagic acid

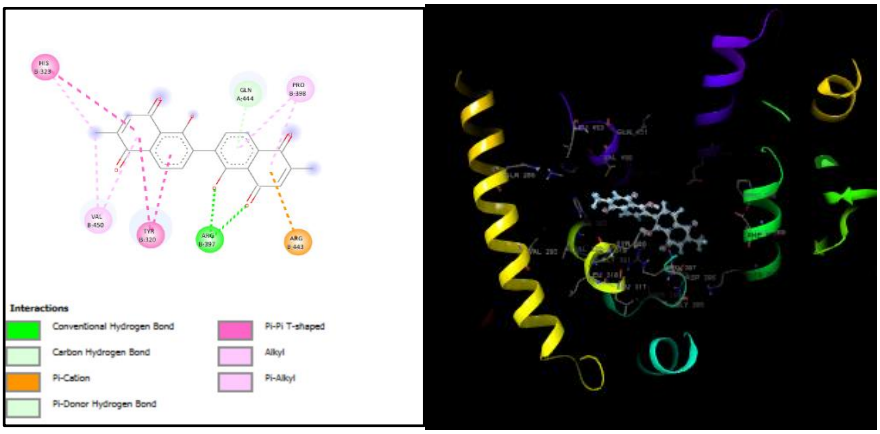


Figure 22. 2D and 3D interaction of 4EMA & Elliptinone

3.6.3. ADME-T Prediction

The computed *in-silico* ADME-T results of the phytocompounds in *Plumbago zeylanica* are presented in Table 7 . These compounds were quickly analysed for Lipinski's rule, and it was found that compounds all these compounds follow Lipinski's rule of five. After that ADME properties of these compounds was checked from SwissADME and found that they fulfil other ADME criteria. Now, toxicity studies were predicted using pkCSM online server and it came out that chitranone is hepatotoxic. Based on the results of the human intestinal absorption (HIA) test, it was found that all the compounds exhibited high permeation across the membrane, indicating good absorption potential. In this analysis, we revealed that Zeylanone, Elliptinone, 8,8'-Biplumbagin, Methylnaphthazarin, Plumbagic acid showed a better pharmacokinetic and hepatotoxicity profile than other compounds. Based on molecular docking study and ADME-T prediction, we concluded that four compounds (Plumbagin, Elliptinone, 8,8'-Biplumbagin, Plumbagic acid) were showed both high binding affinity to various target proteins and good ADME-T profile.

The interactions between the ligand and protein receptor can mainly be hydrophobic or hydrogen bonding. These types of interactions are good because they are not too much hounded or not too loosely bonded to the receptor. Optimized binding is required because drag needs to be eliminated from the body. Too many binding interactions and strong binding can lead to toxicity.

Table 7. Toxicity prediction for the compounds obtained by using pkCSM

Properties	A2	A3	A4	A5	A7	A8	A10
Water solubility (Logmol/L)	-4.18	-1.888	-2.655	-3.575	-4.339	-3.646	-2.354
Caco2 permeability (log Papp in 10-6cm/s)	0.704	0.645	1.192	0.813	0.71	0.422	0.146
Intestinal absorption (% absorbed)	90.23	92.661	96.258	89.019	88.841	95.509	50.223
Skin permeability (log Kp)	-2.74	-2.911	-2.933	-2.74	-2.784	-2.768	-2.73
P-gp substrate	Yes	No	No	Yes	Yes	Yes	Yes
P-gp I inhibitor	Yes	No	No	Yes	No	No	No
P-gp II inhibitor	-4.18	-1.888	No	No	No	-3.646	No
VDss (human)(logL/kg)	-0.189	0.27	0.14	-0.18	-0.29	0.14	-0.341
Fraction unbound (Fu)	0.102	0.491	0.448	0.077	0.06	0.015	0.465
BBB permeability (Log BBB)	0.213	0.079	0.476	0.227	0.047	-0.347	-0.792
CNS permeability(Log PS)	-2.92	-2.254	-2.829	-3.056	-2.924	-2.837	-2.806
CYP2D6Substrate	No	No	No	No	No	No	No
CYP3A4 substrate	No	No	No	Yes	Yes	Yes	No
CYP1A2 inhibitor	No	No	Yes	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	Yes	Yes	No
CYP2C9 inhibitor	Yes	No	No	No	Yes	Yes	No
CYP2D6 inhibitor	No	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	Yes	No	Yes	No
Total Clearance (log ml/min/kg)	0.133	0.05	0.148	-0.04	0.367	0.28	0.231
Renal OCT2 substrate	No	No	No	No	No	No	No
AMES Toxicity	No	Yes	Yes	No	No	No	No
Hepatoxicity	Yes	No	No	No	No	No	No
Skin sensitization	No	No	No	No	No	No	No

4. Discussion

The present study is of great significance as it is the first one to report *in-silico* findings of phytocompounds from *Plumbago zeylanica*. It indicates their potential role as nephroprotective agents. Along with the *in-silico* experiments, *in-vitro* findings of hydroalcoholic extracts of *Plumbago zeylanica* (root, stem, and leaf) showed comparative activity of α-amylase and α-glucosidase inhibition activity and the glucose uptake capacity in yeast.

cells. Antioxidant study was also done by DPPH free radical scavenging activity to investigate the antioxidant activity of hydroalcoholic extracts of *Plumbago zeylanica.* The results of *in-vitro* studies showed that the hydroalcoholic extracts of *Plumbago zeylanica* root have greater inhibition activity and glucose uptake activity than the hydroalcoholic extracts of *Plumbago zeylanica* stem, followed by hydroalcoholic extracts of *Plumbago zeylanica* leaf.

In the current study, plant-derived phytocompounds were used against six target proteins (ALR, ACE, DPP-4, PPAR-α, PPAR-γ and RAGE) to detect their efficacy against diabetic nephropathy. For aldose reductase, the highest binding affinity was found with 8,8'-Biplumbagin and plumbagin, (-9.196, -9.089). For PPAR- α, the highest binding affinity was found with 8-8’ biplumbagin, plumbagin, elliptinone and Plumbagic acid (-8.118, 8.367, -7.467, -7.196). The results of docking analyses were considered satisfactory for drug designing and based on their ADMET profile it suggests that the studied phytocompounds have the potential to be investigated as nephroprotective agents for

diabetic nephropathy. It allows researchers to evaluate various properties, including biodegradation, and aids in the identification of promising compounds for further investigation.

Due to the following limitations: financial constraints, socio-economic obstacles, limited resources etc., the current study was conducted with a small sample size and limited variables were used. Therefore, in future, a study may be performed with a larger sample size, and further in-vivo experiments, which could lead to evaluation of the mechanism of action of nephroprotective effect of plant extracts before proposing it as a new medicine diabetic nephropathy.

5. Conclusion

Through *in-silico* studies, the binding affinity of the phytocompounds with the target protein receptors was determined, providing insights into their potential therapeutic effects in diabetes and diabetic nephropathy. Additionally, the ADMET and drug likeliness properties were assessed, which provided valuable information regarding the pharmacokinetic and safety profiles of the phytocompounds.

Furthermore, *in-vitro* experiments were conducted to evaluate the anti-diabetic and nephroprotective activities of *Plumbago zeylanica*. These experiments provided direct evidence of the plant's potential to alleviate diabetes and protect against nephropathy.

Overall, the findings of our research contribute to the growing body of knowledge regarding the nephroprotective potential of *Plumbago zeylanica*. The *in-silico* and *in-vitro* studies provide a solid foundation for further research and development of therapeutic interventions for diabetes and related kidney complications using natural compounds derived from this plant.

6. References

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