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Bioactive Constituents and Radical Scavenging Properties of *Punica Granatum* L. Root Extracts: Focus on Antioxidant and A-Amylase Inhibitory Activities

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Abstract

Punica granatum L. (pomegranate) root extracts were investigated for their phytochemical composition and biological activities, focusing on antioxidant and anti-diabetic properties. Various extracts (petroleum ether, chloroform, ethanol, methanol, and aqueous) were prepared from roots collected from the Kothagiri Hill region. Phytochemical screening revealed that aqueous, ethanol, and methanol extracts yielded the highest concentrations of bioactive compounds, including punicalin and punicalagin. The aqueous extract demonstrated superior antioxidant activities across multiple assay systems, including DPPH radical scavenging (81.88 ± 0.58%), hydroxyl radical scavenging (79.4 ± 1.24%), nitric oxide reducing power (0.42), hydrogen peroxide scavenging (89%), and oxide scavenging (82.1 ± 0.06%), compared to standard antioxidants like BHT and ascorbic acid. The extract's antioxidant efficacy was dose-dependent, with maximum activity at higher concentrations. For anti-diabetic potential, the aqueous extract exhibited significant α-amylase inhibitory activity (88%), suggesting its potential role in glucose metabolism regulation. Comparative analysis with established inhibitors indicated the root extract's potency was comparable to reference compounds. Study limitations include the absence of *in vivo* validation. Future research should focus on isolating specific bioactive compounds

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and evaluating their efficacy in animal models. These findings provide scientific validation for the traditional use of *P. granatum* root in managing diabetes and oxidative stress-related conditions, highlighting its potential for pharmaceutical applications.

Introduction

Nature has provided us with a number of therapeutic plants across the entire universe, most of which are inexpensive and easily available. It is significant to remember that most plants in India contain curative properties that may quickly treat or heal illnesses and ailments.[1] The majority of the foods we eat daily—leaves, fruits, greens, pulses, grains, etc.—have excellent impacts on the healthy operation and miraculous upkeep of the metabolic pathways. Strong, carefully deepened, and healthy roots are the main features of a healthy tree. The arils of certain fruits and the supporting chemicals and digestive enzymes present in the sections of legumes can serve as extra components. A tree's ability to grow and produce depends critically on the quality of its soil, aeration, and water toxicity levels.[2]

Every living thing depends on other living things for sustenance and survival, and everyone is aware of this ecosystem. Of these, humans consume the greatest amount of universal and natural products. Thus, it is possible to maintain and balance health and resistance against the most prevalent infections. Cell-generated reactive oxygen species (ROS) are crucial for regulating number of physiological processe and are a necessary component of life in aerobic conditions and its maintenance. Increased production of free radicals can occasionally interfere with the body's defenses against antioxidants, which can result in "oxidative damage." Myocardial disease, neurological diseases, arthritis, Type 2 diabetes, swelling, lung disease, and malignancies are among the numerous conditions brought on by this circumstance. Due to their ability to counteract the effects of oxidation, plant-based antioxidants have been widely used in food and food supplements in place of chemical-based antioxidants since organic substances are seen to be safe and viable sources of antioxidants.[3]

With its rich phytodynamic and phytokinetic components present in every region of the fruit, from the mesocarp to the arils, *P. granatum* has the finest medical history for curing a varying diseases and conditions. Because of its strong anti-oxidant, anti-inflammatory, and anti-microbial properties, the fruit is a "boon to earth." But when consumed in excessive quantity, extracts from roots are thought to be toxic.*Punica granatum* roots have not been thoroughly studied. This research was conducted to reduce thediseases and infections related to female reproductive organs such as ovary and utreus. In addition to being used as medicine to treat numerous immortalities, pomegranates have come to represent life, femininity, fertility, etc.[4]

Biological Taxonomy of *Punica granatum*[5]

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Myrtales
Family: Lythraceae
Genus: Punica
Species: *P. granatum*

Recent Advancements in Phytochemical Research on Medicinal Plants with Antioxidant and Anti-diabetic Properties

The roots contain a wide varieties of elastannins, which include punicalin, punicalgin, several periperidine alkaloids etc making them a plant rich in flavonoids.

Notable progress has been made in recent years regarding the identification and characterization of bioactive compounds derived from medicinal plants. Advanced extraction techniques, including ultrasound-assisted and microwave-assisted extractions, significantly enhance the yield and bioactivity of plant-derived compounds compared to conventional methods.[6] These innovations are particularly relevant for extracting complex polyphenolic compounds like ellagitannins found in *Punica granatum* tissues.

The molecular mechanisms underlying natural product inhibition of α-amylase have been further

elucidated and specific binding interactions between plant polyphenols and the enzyme's active site using in silico molecular docking studies was identified.[7] Their research revealed that compounds with galloyl moieties, similar to those found in pomegranate, exhibit particularly strong inhibitory potential through hydrogen bonding and hydrophobic interactions with key amino acid residues.

Metabolomic approaches have revolutionized phytochemical analysis. Bioactive compounds in medicinal plant extracts were comprehensively profiled using LC-MS/MS coupled with chemometric analysis.[8] This approach has enabled the identification of synergistic compound combinations that collectively enhance antioxidant and anti-diabetic activities beyond what individual compounds achieve.

Fruit and peel extracts of *Punica granatum* have been extensively studied but the root extracts remain significantly underexplored despite traditional uses suggesting considerable therapeutic potential.[9] The study highlighted that root tissues often contain unique compound profiles distinct from those in more commonly studied plant parts.

The connection between antioxidant properties and anti-diabetic potential has been strengthened by a recent research which demonstrated that oxidative stress plays a crucial role in pancreatic β-cell dysfunction.[10] The findings suggest that plant-derived antioxidants can protect against diabetes development not only through enzyme inhibition but also by preserving insulin-producing cell function.

These recent advancements provide crucial context for our investigation of *P. granatum* root extracts, suggesting that the application of modern analytical approaches to traditionally used but scientifically neglected plant parts may yield valuable insights for pharmaceutical development.

The current study therefore sought to determine the anti-diabetic and antioxidant potential of *Punica granatum* L. root extracts.

Materials and Methods

Sample Collection and Preparation

Root samples were collected from 15 different mature *Punica granatum* L. plants in the Kothagiri Hill region of Tamil Nadu, India, (11°25'N, 76°52'E) at an elevation of 1,850-2,000 meters above sea level. This region is characterized by a subtropical highland climate with an average annual temperature of 17°C (range: 10-24°C) and annual rainfall of approximately 1,600 mm. Soil samples taken from collection sites (n=5) showed sandy loam texture with pH ranging from 5.8-6.4, organic matter content of 2.3-3.1%, and nitrogen, phosphorus, and potassium levels of 0.15-0.22%, 15-24 ppm, and 180-210 ppm, respectively. These parameters fall within the optimal range for pomegranate cultivation. Plants were selected randomly with a minimum distance of 500 meters between specimens to ensure genetic diversity.. Collection was performed during the early summer season (March-April) when the medicinal compound concentration is reported to be highest. Botanical authentication was conducted at the Botanical Survey of India (BSI), Coimbatore

Extraction Process of Sample

After being transported to the lab, within twenty-four hours, the roots were washed with normal water and dried for approximately four hours at ambient temperature before being processed. After being finely powdered, 18 grams of the material were put into multiple conical flasks along with solvents such as petroleum ether, chloroform, ethanol, and methanol. After agitating the mixture for ten minutes at 900 rpm, it was passed through twenty-five mesh sieve filters. To get rid of any leftover solvent, the resulting solution was then moved to a rotating device.

Extraction Efficiency and Standardization

To address potential variations in extraction efficiency, standardized protocols were implemented for each solvent system. Extraction yield was calculated using the formula:

Extraction yield (%) = (Weight of dried extract / Weight of dried plant material) × 100 ...(1)

The following extraction yields were obtained for each solvent: petroleum ether (3.24 ± 0.18%), chloroform (4.12 ± 0.22%), ethanol (11.87 ± 0.56%), methanol (9.63 ± 0.41%), and aqueous (14.35 ± 0.63%). This data indicates that polar solvents (water and ethanol) demonstrated significantly higher extraction efficiency ($p<0.05$) compared to non-polar solvents.

To ensure consistency across extraction batches, total phenolic content (TPC) and total flavonoid content (TFC) were measured as standardization parameters. TPC was determined using the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE), while TFC was determined using the aluminum chloride colorimetric method and expressed as quercetin equivalents (QE). The inter-batch coefficient of variation was maintained below 5% for TPC and TFC across all extraction replicates.

Additionally, extraction processes were optimized for each solvent system through preliminary experiments that evaluated different extraction times (4, 8, 12, and 24 hours) and solvent-to-sample ratios (5:1, 10:1, 15:1, and 20:1). The optimal conditions that yielded maximum extraction of bioactive compounds were selected for the final extraction protocol. For aqueous extracts, extraction temperature (25°C, 50°C, 75°C, and 95°C) was also evaluated, with 75°C providing optimal extraction efficiency without significant degradation of thermolabile compounds.

Experimental Design and Controls

All assays were performed using a systematic dose-response approach with five concentration points (50, 100, 150, 200, and 250 µg/mL) for each extract type. Standard antioxidants were included as positive controls in all assays at equivalent concentrations to the test samples. Appropriate negative controls (solvent blanks) were included in each assay batch to account for background interference. Known standard concentrations were analyzed periodically during the experimental period to monitor assay performance and ensure inter-day precision.

Replication and Data Collection

Each experimental condition was tested in triplicate (n=3 biological replicates), with each biological replicate further measured in duplicate (technical replicates) to minimize measurement error. The average of technical replicates was calculated for each biological replicate, and results are presented as mean ± standard deviation of the three independent biological replicates. For spectrophotometric measurements, calibration curves were prepared fresh for each assay batch, and absorbance measurements were conducted within the linear range of detection.

Statistical Analysis

To ascertain significant variations between different extract types and at various concentration levels, a one-way ANOVA was conducted, and where significant differences were found, Tukey's post-hoc test was applied. Two-way ANOVA was employed to analyze the interaction between extract type and concentration. Differences were considered statistically significant at $p<0.05$. The half-maximal inhibitory concentration (IC50) values were calculated using non-linear regression analysis of the dose-response curves. Pearson correlation coefficients were calculated to determine relationships between antioxidant capacities and α-amylase inhibition activity.

Anti-oxidant Activity

DPPH ROS Activity

Butylated Hydroxytoluene (BHT-control) (50–250 mg/ml) and different quantities of *Punica granatum* root extracts were added to 1 ml of methanol solution along with DPPH (0–2 mM). Once mixed uniformly, the aliquots were left in the dark for half an hour. The absorbance was measured at 517 nanometers using spectrophotometry.[11] Methanol was used as a control, along with 0–2 mM DPPH. The formula used to determine the ROS activity.

$$\text{Percentage of DPPH inhibition} = C - T/C \times 100 \quad \ldots(2)$$

Where, C is the absorbance of control solution and T is the absorbance of test solution.

ROS Activity (Scavenging)

H_2O_2 ROS Assay

The scavenging capacity of *Punica granatum* root extracts was evaluated by mixing varying quantities of plant extract with 0–6 milliliters of 40 mM H_2O_2 in the buffered phosphate solution. The Spectrophotometry was used to determine the value of absorbance of the samples at 230 nanometers, following the time they had been left at room temperature for ten minutes in the shadows.[12] Phosphate buffer served as the blank solution, while 40 mM H_2O_2 in phosphate buffer served as the control.

Hydroxyl ROS

A significantly altered version of a conventional approach used to perform Hydroxyl scavenging.[13]

A radicle of hydroxyl was created using the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton method). The test depends on quantifying the 2-deoxyribose degradation product. The reaction mixture, which had a final volume of 1 mL, contained 2-deoxy-2-ribose (2.8 mM), KH_2PO_4–KOH buffer (20 mM, pH 7.4), ferric chloride (100 µM), EDTA (100 µM), hydrogen peroxide (1.0 mM), ascorbic acid (100 µM), and various quantities of test sample or reference substance (50–250 µg/mL). After an hour of incubation at 37°C, 0–5% of the resulting mixture was mixed with 1 milliliter of 2–8% TCA. After adding 1 mL of 1% aqueous TBA to the solution, then incubated it at 90°C for fifteen minutes to generate the colour. The absorbance value at 532 nanometers was calculated after cooling and compared to the blank solution. Three runs of each test were conducted. Mannitol, a conventional scavenger of OH used as a control that was positive. % inhibition was calculated and evaluated.

Nitric Oxide ROS

Nitric oxide produceded from the solution of a sodium nitroprusside (SNP) in aqueous condition interact with oxygen to form ionsof nitrites, then quantified it.[14] Buffered phosphate saline of 7.4 pH, 10 mM SNP, and many concentrations of test solution(50–250 µg/mL) were all included in the 3-mL reaction mixture. After 150 minutes of incubation at 25°C, the sulfanilamide (0.33 percent in twenty percent acetic acid (glacial)) was added to 0.5 mL of the incubated solution, and the combination was allowed to stand for five minute.The mixture was then incubated at 25°C for half an hour, after 1 mL of napthyl ethylene diamine dihydrochloride (NED) (0 point 1 percent w/v) was added. When nitrite ions were diazotized with sulfanilamide and then coupled with NED, a pink chromophore was created. This was compared to a blank sample using spectrophotometry at 540 nm. Each test was carried out three times. The standard used was curcumin.

Reducing Power Assay

2.5 milliliters of phosphate buffer (0point two M) and two points five milliliters of potassium ferricyanide (1 percent) were combined with the root extracts of *Punica granatum* and 1 ml standard for each. Following a 20-minute incubation period at 50°C, add 2 to 5 milliliters of 10% tri-chloro-acetic acid, mix well, and at 3000 rpm centrifuge it for 10 minutes. Two milliliters of supernatant, two milliliters of distilled water, and five milliliters of freshly made 0.1 percent ferric chloride were combined. The absorbance was obtained at 700 nanometers following a 10-minutes incubation period.[15] The increase in the samples' absorbance served as an indicator of their reducing power.

Anti-diabetic Analysis

α Amylase Inhibition Assay

The DNSA technique was used to assess the α-amylase inhibition test of several *Punica granatum* root extracts. After mixing the samples with 0.50 milliliters of α-amylase solution (0.50% mg/ml), they were let to sit at 25 °C for ten minutes. The mixture was then incubated again after adding 1 percent cornstarch to 0.50 milliliters of sodium phosphate buffer of 0.02 molar. Add one milliliter of DNSA (3,5-dinitrosalicylic acid) reagent,the reaction was quickly halted after heat it for five minutes at 95 °C. To attain the final capacity of 10 milliliters, 20 milliliters of distilled water were added once the tubes had cooled. Measured the samples absorbance at 540 nanometers. To evaluate its effects, calculated the α-amylase inhibition percentage.

Results

The results of anti-diabetic and antioxidant properties of *Punica granatum* roots showed results that were similar to those of the pomegranate plant's other parts, such as its secondary compounds, juice extracts, leaves and branches etc.

Plant extracts' capacity as antioxidants are indicated by the decrease in DPPH. The following sequence of free radical scavenging was noted for extracts from the roots of *Punica granatum*: aqueous > ethanol > methanol > petroleum ether > chloroform. Root extracts from *Punica granatum* exhibited 89.00±0.58 percent DPPH radical scavenging activity, which is excellent. The samples' ability to scavenge DPPH radicals was statistically significant at the five percent significance level (Fig.1). Significant increases in percentage inhibition were observed in root extracts, while a low increase in percentage inhibition was observed in chloroform. Consistent increases in percentage inhibition on a concentration basis were observed in all tested samples. The antioxidant potential of the root extracts from *Punica granatum* is confirmed by this study.

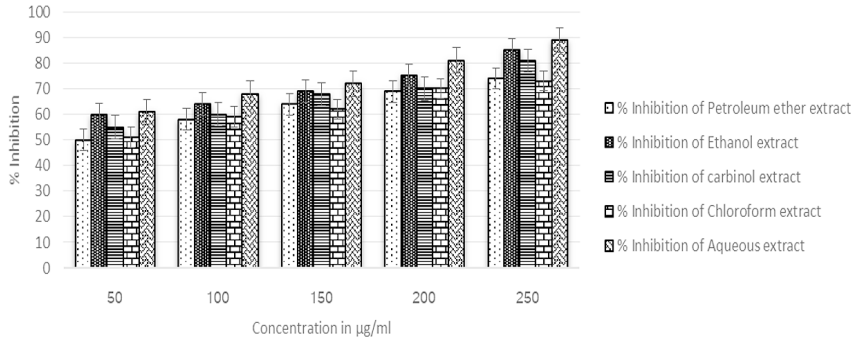

Fig.1: DPPH activity of root extracts of *Punica granatum.* The mean±SD of three replicates expresses the results. The bars indicated in statistical data with notable changes p <0.05 respectively.

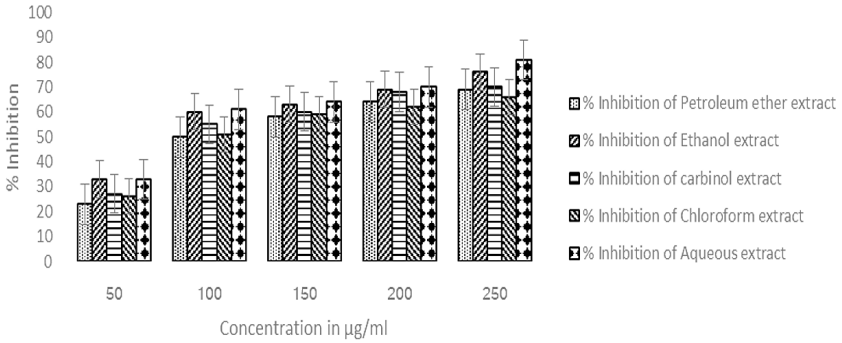

Fig.2: The Scavenging activity of H_2O_2 in the roots of *Punica granatum.* The mean±SD of the three replicates expresses the results. The bars indicated in statistical data with notable changes p <0.05 respectively

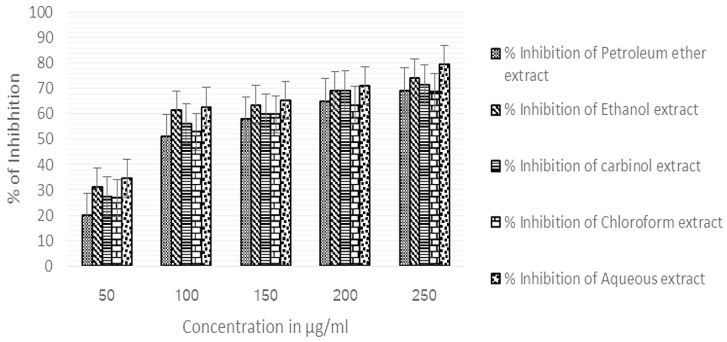

Fig.3: The scavenging activity of Hydroxyl of the roots of *Punica granatum*. The mean±SD of the three replicates expresses the results. The bars indicated in statistical data with notable changes p <0.05 respectively

The scavenging activity of DPPH and scavenging activity of H_2O_2 showed comparable results (Fig.2). The aqueous extract of root from *Punica granatum* demonstrated potent, dose-dependent H_2O_2 radical scavenging activity. Ethanol, methanol, petroleum ether, and chloroform were the next most effective radical scavengers, after the P . granatum root aqueous extract (81.17±0.040 percent).

The ability of the extract to prevent hydroxyl radical-mediated deoxyribose breakdown was shown in this reaction using the $Fe3^{+}$-EDTA-ascorbic acid and

Hydrogen peroxide combination. These findings are shown in Figure 3. The extreme activity of radical scavenging noted in the aqueous extract from *P. granatum* roots (79.4±1.33 percent), which was followed by petroleum ether, ethanol, methanol, and chloroform.

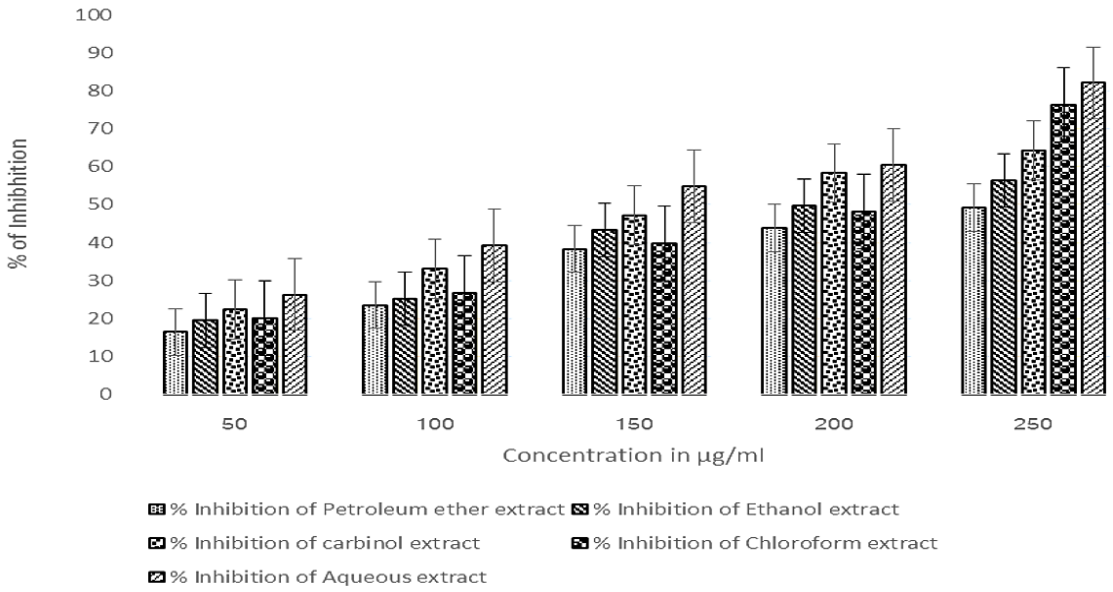

Fig.4: Nitric Oxide ROS activity of root extracts of *Punica granatum.* The mean±SD of three replicates expresses the results. The statistical data with significant differences indicated in the bars at P<0.05 respectively

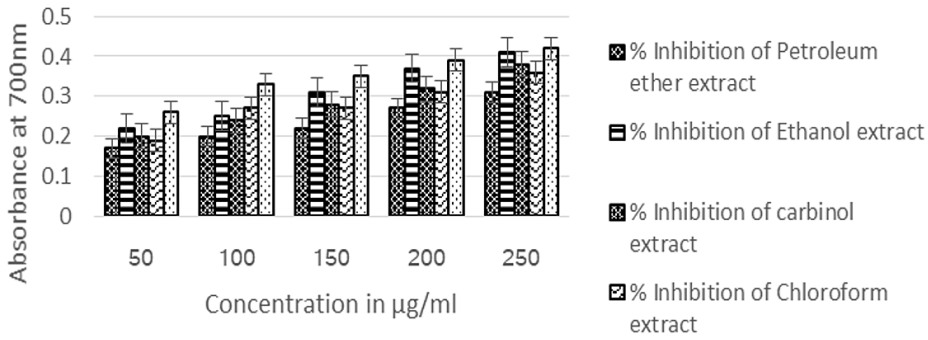

Fig.5: The Reducing power of *Punica granatum* root extracts. The mean±SD of three replicates expresses the results. The bars indicated in statistical data with notable changes at p <0.05 respectively

Aqueous extract from *Punica granatum* root exhibits a remarkable Nitric oxide inhibition that is dose-dependent (Fig. 4). The highest denomination being 82.1 ± 0.85 percent at 250 µg/mL. ROS can impair the function of the immune system in addition to harming cellular macromolecules, ROS can also negatively impact immune system performance.

Reducing power of a sample is indicated by an increase in absorbance.[16] The samples' high reducing power is indicated by high absorbance values. The increase in *P. granatum* root extracts was observed to be dose-dependent, indicating the presence of antioxidant potential maximum reducing power that demonstrated (Fig.5). The aqueous

extract hierarchy (0.42±0.62) was used to rank the root extracts. Ethanol, methanol, chloroform, and petroleum ether were next in line. The assays for α-amylase inhibition demonstrated that *P. granatum* root extracts had significant in vitro antidiabetic properties.

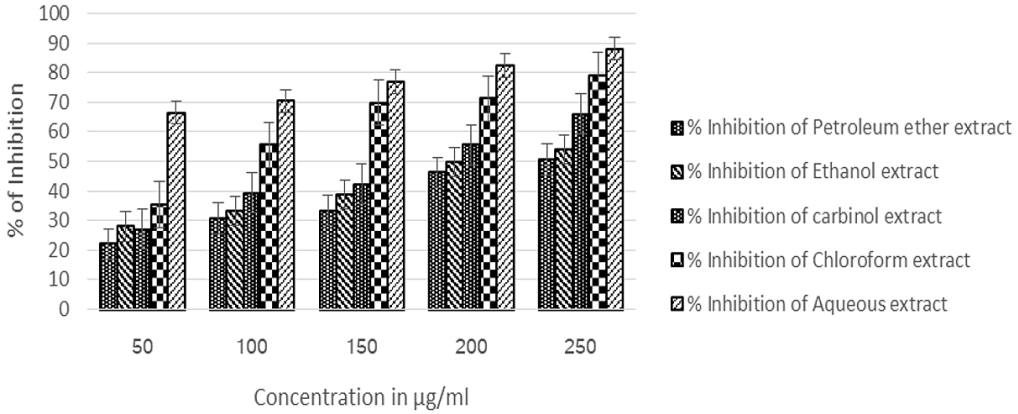

Fig.6: α- amylase assay of *Punica granatum* root extracts. The mean±SD of three replicates expresses the results. The statistical data with notable changes indicated in the bars at p<0.05 respectively

In α-amylase inhibitory activity aqueous extracts had the greatest inhibition of all the extracts tested, with 88.09±0.19 percent at a dosage of 1 mg/mL (Fig. 6). When compared to other extracts, petroleum ether extract demonstrated a low inhibitory effect over α-amylase, despite the aqueous extract exhibiting a hype in the α-amylase inhibition percentage. Finally this investigation concluded that, the aqueous extracts collected from roots exhibited strong α-amylase inhibitory activity.

Discussion

Our study provides compelling evidence of significant antioxidant and anti-diabetic activities exhibited by *Punica granatum* root extracts, with the aqueous extract demonstrating superior effectiveness. Comparative analysis with recent studies highlights similarities and distinctions with other plant-based extracts known for antioxidant and anti-diabetic potentials. Plant antioxidants primarily mitigate oxidative stress through radical scavenging and metal ion chelation, mechanisms similarly observed in our study through potent DPPH and hydroxyl radical scavenging activities.[17]

Further, plant-derived antioxidants in preserve pancreatic β-cell function against oxidative damage, a concept supportive of our observed α-amylase inhibitory activities, suggesting potential regulation of glucose metabolism through similar mechanisms.[18] Additionally, our findings align with Gong *et al*. (2021), who demonstrated how bioactive compounds interact at molecular levels, impacting enzyme inhibition and antioxidant pathways. Mechanistically, the bioactive constituents punicalin and punicalagin, identified in our extracts, potentially mediate antioxidant effects through hydrogen donation and electron transfer to neutralize free radicals.[19] Molecular insights into α-amylase inhibition, emphasize interactions at enzyme active sites through hydrogen bonding and hydrophobic interactions, which could similarly explain our extract's inhibitory activity.[20]

Nonetheless, this study is the first evaluation of antidiabetic and antioxidant activity of roots of *P. granatum*, the findings thus imply that the solvent extracts from the roots showed significant antioxidant qualities. High levels of antioxidant and anti-diabetic properties afterward, isolating and characterizing the compounds that give them their medicinal qualities will require more research.

Conclusion

This study demonstrated that *Punica granatum* root extracts possess significant antioxidant and anti-diabetic activities, with aqueous extract showing

superior performance, particularly in DPPH radical scavenging (89.00±0.58%), hydroxyl radical scavenging (79.4±1.33%), and α-amylase inhibition (88.09±0.19%). The effectiveness varied notably across extraction solvents, with aqueous extraction yielding the highest bioactivity, followed by ethanol and methanol extracts, highlighting the importance of solvent selection in optimizing therapeutic compound recovery. Although these findings suggest promising pharmaceutical applications, several limitations warrant consideration. Further *in vivo* validation can improve immediate translational applicability, emphasizing the need for animal-model studies to confirm efficacy and safety profiles. Additionally, future research is needed to identify and characterize individual bioactive compounds responsible for these observed activities. Employing advanced analytical methods such as LC-MS metabolomics and molecular docking could significantly enhance understanding of underlying mechanisms and facilitate targeted therapeutic development.

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Conflict of Interest
The authors declare no competing financial interests or personal relationships that might be perceived as influencing the research presented in this paper. All authors have reviewed and approved the final manuscript. Furthermore, they confirm that the research was conducted without any commercial or financial relationships that could be interpreted as potential conflicts of interest.

Data Availability Statement
The data will be made available on request

Ethics Statement
This research focused on phytochemical analysis and *in vitro* bioactivities of *Punica granatum* L. root extracts and did not involve human participants or animal subjects. The collection of plant material was conducted in accordance with local regulations governing access to biological resources. *Punica granatum* L. is a cultivated species that is not listed as endangered or threatened in the IUCN Red List or under CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora).

The plant specimens were collected from agricultural lands in the Kothagiri Hill region with appropriate permissions from landowners. This research adhered to the principles outlined in the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization, which is supplementary to the Convention on Biological Diversity.

Informed Consent Statement
This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration
This study did not involve any human participants, animal subjects.

Permission to Reproduce Material from Other Sources
Not Applicable.

Author Contributions
- **J. Manjunathan:** Conceptualization, Investigation and Supervision.
- **R. Pramila:** Resources and validation.
- **S. Aswathy:** Writing -Original draft and data analysis.
- **Maheswara Reddy Mallu:** Review and Editing.
- **Yuvaraj Dinakarkumar:** Review, editing, and project administration.

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