Integrating phytochemical profiling, in vitro studies and network pharmacology to reveal the antidiabetic action mechanism of *Punica granatum* fruit peel

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**ABSTRACT:** Despite considerable studies, diabetes continues to affect millions of people worldwide with an increasing rate of morbidity and mortality. Therefore, we aimed to explore the *in vitro* antidiabetic and antioxidant effects of *Punica granatum* and also reveal its drug-likeness, toxicity and action mechanism by utilizing SwissADME (absorption, distribution, metabolism and excretion), ProTox-II and network pharmacology, respectively. The results showed that *P. granatum* has a significant dose-dependent inhibition potential against *α*-amylase (*IC*$_{50}$=131.90 ± 0.44) and *α*-glucosidase (*IC*$_{50}$=149.74 ± 0.58) activity, as well as a significant inhibition in DPPH-free radical scavenging (*IC*$_{50}$=108.57 ± 0.52) activity. ADME/Toxicity prediction of the compounds satisfies Lipinski’s rule of five with zero violations and did not find any toxicity. Moreover, network pharmacology revealed that polyphenolic compounds of *P. granatum* may combat diabetes by acting on key targets such as IRS1, TNF-α, IL6, MAPK3, DPP4, LEPR, GSK3B, PPARA and PIK3CG were strongly involved in glucose metabolism, oxo-inflammatory responses and insulin-related pathways. Based on the finding, we can conclude that *P. granatum* might be promising therapeutics for the management of diabetes and related disorders.

1. **INTRODUCTION**

Diabetes mellitus, also known as diabetes, is a fatal disease characterised by elevated blood glucose levels in the circulatory system (AMA, 2021). Globally, about one in eleven adults suffering from diabetes and 536.6 million people were estimated to be living with diabetes in 2021, which is expected to rise to 783.2 million in 2045. Further, about 541 million people with impaired glucose tolerance are at a high risk of developing diabetes, and the global healthcare expenditure was estimated to be USD 966 billion in 2021 (AMA, 2021). High prevalence, variable pathogenesis, and complications of diabetes warrant the urgent need for its effective management.

One remedial method of treating diabetes is to inhibit/block glucose absorption by inhibiting the enzymes *α*-amylase and *α*-glucosidase (Gaurav et al., 2020). Salivary and pancreatic *α*-amylase cleaves the starch into simple saccharides at random sites and forms smaller molecules of glucose that are absorbed into the bloodstream. The *α*-amylase inhibitors prevent starch from being converted into simple sugars or slow sugar absorption in the gastrointestinal tract. *α*-Glucosidase is an exo-type carbohydrate found in many microorganisms, plants, and animal tissues that catalyses the release of glucose from the substrate's non-reducing end. It is a membrane-bound enzyme found in the small intestine’s brush border membrane that works to facilitate glucose absorption by catalysing the hydrolytic cleavage of oligosaccharides into absorbable monosaccharides (AMA, 2021; Lawal et al., 2022).

WHO reported that more than 80% population of the world in developing countries uses herbal medicine to combat the progression of various diseases and disorders. In spite of conventional antidiabetic medicines, numerous pharma companies focus to developed herbal medicine that controls the progression of diabetes and related disorders (Osman et al., 2019; Tugume & Nyakoojo, 2019). The potential of plants as a source for the production of new drugs is largely untapped *Punica granatum* fruit peel is one of them. It is frequently consumed throughout the world. *P. granatum* majorly contains polyphenols like quercetin and gallic acid, and these are the compounds responsible for antioxidant, liver protection, insulin secretion, cardioprotection, gut health, cancer prevention and many more (Faddladdeeen & Ojaimi, 2019; Lawal et al., 2022; Middha et al., 2013). Moreover, previous experimental studies revealed that the mode of diabetes management by utilizing

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polyphenols has been attributed to their ability to hinder the digestion of carbohydrates by inhibiting the enzymes α-amylase and α-glucosidase. Hence, polyphenols may play a promising enzyme inhibitory compounds to manage diabetes. To date, no prior reports were found on the role and its action mechanism of polyphenolic compounds of *P. granatum* for diabetes management.

Biomedical scientists now recognize that the "one key, one lock" hypothesis is insufficient for deciphering drug actions, particularly in complex diseases (Zhou et al., 2019). Intriguingly, for fast screening of bioactive compounds in the mixture of plant-based products computer-aided techniques are useful to explain or predict the pharmacological effect of a drug. Moreover, computational methods of pharmacological screening of lead compounds reduce the cost of the experiment, time-consumption in discovery and most importantly minimize the number of investments and frillier at any stage of drug development of bioactive leads from a huge loss to the organization (Huang et al., 2020; H. Li et al., 2014). However, network pharmacology, which analyses drugs and drug targets systemically, may provide us with profound insight into drug actions. It is now widely accepted as a valuable tool for assessing systemically and illustrating the rationality of drugs. Keeping in view, the present study aims to explore the enzyme inhibitory potential of *P. granatum* and also reveal drug-likeness, toxicity and action mechanism by utilizing SwissADME (absorption, distribution, metabolism and excretion), ProTox-II and network pharmacology, respectively.

2. MATERIALS AND METHODS

Fresh peels of *P. granatum* were purchased and authenticated (voucher specimen number; A/06-22/350) in-house at Abhinav Healthcare Product Private Limited, Mumbai, India. The dried peel was coarse powder and transferred in a round bottom flask containing water and alcohol (30:70) for extraction. After the process, filtered the obtained hydroethanolic extract and concentrated to dryness using a rotary evaporator and stored the process, filtered the obtained hydroethanolic extract and concentrated to dryness using a rotary evaporator and stored the process, filtered the obtained hydroethanolic extract and concentrated to dryness using a rotary evaporator and stored.

2.2. HPLC analysis of quercetin and gallic acid

HPLC was used to identify polyphenolic compounds in *P. granatum* using standard markers (quercetin and gallic acid) in the samples according to the described methods (Gini & Jothi, 2018). Before use, the mobile phase underwent ultrasonic degassing and a nylon membrane filter (0.2 µm pore size, Millipore, Merck, Germany). For quercetin and gallic acid, the lambda max was 254 nm and 278 nm, respectively. For quercetin, methanol (A) and 4% acetic acid (B) was used as solvent system whereas, for gallic acid, the solvent system was composed of acetonitrile (A) and 0.1% phosphoric acid in water (B). The 1.0 mL/min flow rate was set for the extract and standards. The overall run time was set to 20 min.

2.3. Antioxidant activities

The extract’s ability to scavenge DPPH (2,2-diphenyl-1-pirclylhydrazyl) was tested according to the defined protocol. The samples (10-250 µg/mL) were mixed with a 0.1 mM DPPH solution and left to incubate at 37°C in the dark for 30 min. Thereafter, measured the absorbance at 517 nm to determine the scavenging potential. The reference compound was utilized as quercetin and IC_{50} values were calculated using Microsoft Excel (Fahim et al., 2019).

2.4. Determination of α-amylase and α-glucosidase inhibition potential

Using various concentrations of extract (10-250 µg/mL), the ability to inhibit α-amylase and α-glucosidase was tested. For α-amylase, 1.0 mL of extract (each concentration) and 1.0 mL amylase enzyme were combined and kept at 37°C for 30 min, then 1.0 mL starch solution was added and kept again for 1 hour. In addition, 100 µL of supernatant was used to assess the inhibitory activity using a glucose reagent. For α-glucosidase, 120 µL of various concentrations of extract (10-250 µg/mL) and 20 µL of glucosidase were poured in phosphate buffer saline (PBS) at 37°C for 15 min. The reaction was started by mixing 20 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (prepared in PBS) and incubated for 15 min. After stopping the reaction with 80 µL of sodium carbonate (prepared in PBS), the absorbance at 405 nm was measured. As a positive control, acarbose was used and IC_{50} values were calculated using Microsoft Excel (Gaurav et al., 2020).

2.5. ADME/Toxicity analysis of the lead compounds

The major polyphenolic compounds were identified by utilizing literature and a database. Furthermore, the drug-likeness and toxicity characteristics of selected compounds were analyzed using SwissADME and ProTox-II. ADME/Toxicity
properties determine the drug-like activity of ligand molecules based on Lipinski’s rule of five. PubChem (https://pubchem.ncbi.nlm.nih.gov/) was utilized for the identification of the SMILES (Simplified Molecular Input Line Entry System) of compounds. Subsequently, target information of active compound ingredients in 

subsequently, target information of active compound ingredients in P. granatum was obtained from the database (https://lsp.nwu.edu.cn/). The gene names of the targets were obtained from the UniProt database, and the target names were imported into it with the species set to ”Homo sapiens” (http://www.uniprot.org/).

2.6. Target genes related to selected compounds and diabetes mellitus

Based on the compound SMILES, associated target genes were chosen using Swiss Target Prediction (STP) in "Homo Sapiens" mode (http://www.swisstargetprediction.ch). DisGeNET (https://www.disgenet.org/search) identified diabetes-related genes.

2.7. GO and KEGG enrichment analysis of target proteins

Enrichr was used to analyse the Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of proteins involved in the protein-protein interaction (PPI) network to elucidate the role of target proteins that interact with the bioactive compounds (quercetin and gallic acid) of P. granatum in gene function and signalling pathway (ShinyGO v0.741). The target proteins and pathways involved in the biological process (BP) were also described.

2.8. Protein-protein interaction (PPI and gene-disease association network to unveil action mechanism

The target proteins of related P. granatum ingredients compounds were uploaded to Network Analyst software to construct the PPI to explain the interaction between target proteins. The website calculated a mutual information score for the target proteins. The higher the score, the greater the degree of certainty in the interaction between target proteins. As a result, in the current study, we chose high-confidence data to assure the accuracy of this analysis. In addition, we have also constructed the network between disease and important genes involved in the pathology of diabetes and its associated disorders.

3. RESULTS AND DISCUSSION

3.1. Estimation of total phenolic and flavonoid content

The calibration curves of gallic acid (\( r^2 = 0.9929 \)) and quercetin (\( r^2 = 0.9901 \)) were used to calculate total phenolic and flavonoid content. The total phenolic and flavonoid content per gram of gallic acid and quercetin was found to be 67.57 ± 0.20 and 85.72 ± 0.34 mg equivalents, respectively. Our findings showed that P. granatum is high in phenolics and flavonoids.

3.2. Identification of quercetin and gallic acid HPLC

HPLC analysis of P. granatum revealed the presence of gallic acid (RT: 4.183 min) and quercetin (RT: 13.171 min). Corresponding RT (retention time) of the P. granatum extract was compared and matched with standard compounds gallic acid and quercetin (Figure 1). Gallic acid and quercetin are the most common phytoconstituents present in medicinal plants. Qualitative analysis of these phytoconstituents is getting more important for quality control and based on their chromatographic reports, we can assure their pharmacological potential.

Polyphenols (gallic acid and quercetin), which are abundant in plants, may have a beneficial effect on hyperglycemia by inhibiting the activity of carbohydrate digesting enzymes (\( \alpha \)-amylase and \( \alpha \)-glucosidase), stimulating insulin secretion from pancreatic beta-cells, and decreasing blood glucose levels. Moreover, the cited studies demonstrated that polyphenolic compounds protected beta-cells and their integrity by modulating hyperglycemia and oxidative stress (Jiang et al., 2017). The identification of gallic acid and quercetin in the present work strongly supported the pharmacological significance of polyphenolic compounds for their antioxidant and antidiabetic potential in P. granatum.

3.3. Free radical scavenging assay

DPPH free radicals scavenging ability of extract was calculated as percentage inhibition. In the present study, DPPH screening of P. granatum had clearly shown the dose-dependent antioxidant activity at the different concentrations (10-250 \( \mu \)g/mL). At maximum tested concentration, the inhibition potential of P. granatum and quercetin was 78.65% and 91.24%, respectively. The obtained results revealed antioxidant inhibition by the P. granatum is similar to the reference compound. The overall data was presented in Table 1.

The DPPH evaluation is a simple and reliable procedure to assess the antioxidant properties of herbal products. P. granatum...
3.4. In vitro α-amylase and α-glucosidase inhibition activity

*P. granatum* was tested against the carbohydrates digesting enzyme α-amylase and α-glucosidase and its inhibitory potential were found in a concentration-dependent manner, implying its potential role in diabetes management (10-250 μg/mL). In the case of α-amylase, the IC₅₀ value was found 131.9 ± 0.44 μg/mL whereas in the case of α-glucosidase IC₅₀ value was found 149.74 ± 0.58. The overall data were presented in Table 1.

One treatment strategy for diabetes management is to hinder/block the absorption of glucose in the gastrointestinal tract and also maintain glucose metabolism in the liver by inhibiting the carbohydrate-metabolizing enzymes α-amylase and α-glucosidase (Choudhury et al., 2018; Deshpande et al., 2008). Likewise, in the current study, α-amylase and α-glucosidase inhibitory activity of *P. granatum* is most likely to be due to polyphenolic compounds. Furthermore, there has been substantial scientific studies evidence suggesting that inhibition of α-amylase and α-glucosidase by polyphenols makes it possible to ameliorate hyperglycemia to combat the occurrence of diabetes (Parveen et al., 2020).

### 3.5. ADME/Toxicity analysis

Server-based ADME/Toxicity analysis provides rapid results that could be useful for the development of lead compounds (Hari, 2019). In our study, we utilized Swissdock for ADME analysis of the compounds. Table 2 summarises the obtained results, and Figure S1, Appendix A depicts the docking figure. The predicted properties of both the polyphenolic compounds are satisfying all the five rules of Lipinski’s and it is considered drug-like potential. All the analysed compounds demonstrated good solubility and absorption in the human intestine. During the design of the drug molecule, it is necessary to predict the situation and movement of a drug in the human body. The bioavailability radar provides a quick assessment of a molecule’s drug-likeness by taking into account six properties i.e. lipophilicity (LIPO), SIZE, polarity (POLAR), insolubility (INSOLU), instasuration (INSATU), and flexibility (FLEX). Both the compounds are expected to be orally bioavailable (low flexibility and polarity), less toxic, and absorb well. Toxicity classes ranging from [1 (toxic) to 6 (non-toxic)] revealed that both compounds are safe (Table 2). Toxicity near one is considered more to human health and vice versa. Interestingly, ADME/Toxicity properties of drugs play an important role in drug filtering during the early stages of drug development (Yi et al., 2018).

### 3.6. Target genes and disease association network

In this study, we created a target-disease interaction network using NetworkAnalyst to better understand the relationship between target proteins and disease association (Figure 2). A total of 31 proteins were screened to describe the pathways involving diabetes and its associated disease. These target proteins were found to be primarily involved in diabetes mellitus, insulin resistance, impaired glucose intolerance, hyperglycemia, hyperinsulinaemia, and obesity. According to network analysis, multiple target proteins exist in one pathway, and the same target protein exists in multiple pathways. In essence, the interaction between one target protein and multiple pathways is more significant than the interaction.

**Table 1**

Antioxidant and antidiabetic activity of *P. granatum.*

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>DPPH activity</th>
<th>α-Amylase</th>
<th>α-Glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quercetin</td>
<td><em>P. granatum</em></td>
<td>Acarbose</td>
</tr>
<tr>
<td>10</td>
<td>15.68 ± 0.09</td>
<td>13.25 ± 0.14</td>
<td>9.08 ± 0.08</td>
</tr>
<tr>
<td>25</td>
<td>27.58 ± 0.18</td>
<td>28.32 ± 0.22</td>
<td>16.3 ± 0.32</td>
</tr>
<tr>
<td>50</td>
<td>46.36 ± 0.30</td>
<td>42.48 ± 0.16</td>
<td>39.10 ± 0.19</td>
</tr>
<tr>
<td>100</td>
<td>71.34 ± 0.72</td>
<td>60.89 ± 1.21</td>
<td>64.20 ± 0.32</td>
</tr>
<tr>
<td>250</td>
<td>91.24 ± 0.63</td>
<td>78.65 ± 1.06</td>
<td>89.09 ± 0.78</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>85.50 ± 0.34</td>
<td>108.57 ± 0.52</td>
<td>107.01 ± 0.38</td>
</tr>
</tbody>
</table>
Table 2
Swiss ADME/Toxicity profiling of selected polyphenolic compounds of *P. granatum*.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of metabolites</th>
<th>Lipinski Violation</th>
<th>Bioavailability score</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>Toxicity class</th>
<th>Ali Class</th>
<th>GI absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quercetin</td>
<td>0</td>
<td>0.55</td>
<td>159</td>
<td>3</td>
<td>Soluble</td>
<td>High</td>
</tr>
<tr>
<td>2</td>
<td>Gallic acid</td>
<td>0</td>
<td>0.56</td>
<td>2000</td>
<td>4</td>
<td>Soluble</td>
<td>High</td>
</tr>
</tbody>
</table>

Figure 3. GO biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of potential target genes of polyphenolic compounds. The colour from blue to red indicates the increasing fold enrichment value, the greater the value, the higher the significance of the pathway.

between multiple target proteins and a single pathway. These findings imply that the potent pharmacological components of *P. granatum* may act on these signalling pathways to alleviate the levels of insulin, glucose, inflammation, and oxidative stress associated with diabetes and other related diseases (Amri et al., 2020; Tang et al., 2018).

3.7. GO analysis and KEGG enrichment analysis of target genes

The biological process findings indicated that these targets were involved in the metabolism, insulin response, reactive oxygen species, and most significantly, cytokines-mediated signalling pathways, among other biological processes. Whereas, KEGG pathway enrichment signalling pathways are involved in various signalling pathways such as insulin resistance, diabetes, and non-alcoholic fatty liver disease. Hence, in the current study, we predicted that polyphenolic compounds in *P. granatum* could act on insulin receptor substrate-1 (IRS1), tumour necrosis factor-α (TNF-α), interleukin 6 (IL6), mitogen-activated protein kinase 3 (MAPK3), dipeptidyl peptidase 4 (DPP4), leptin receptor (LEPR), glycogen synthase kinase-3 beta (GSK3B), peroxisome proliferator-activated receptor-α (PPARA) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PIK3CG) are other proteins to achieve an antidiabetic effect. At the same time, it may regulate
Figure 4. Protein-protein interactions of diabetes-related genes and polyphenolic compounds quercetin and gallic acid.

Figure 5. Correlation between polyphenolic compounds (cyan rectangles) and diabetes-related genes (round) and their regulated interactive pathway network of P. granatum.

Multiple signalling pathways related to insulin resistance, glucose metabolism, and oxo-inflammations, and achieve the purpose of restoring glucose homeostasis and helping the body return to normal (Figure 3).

3.8. PPI network of targeted genes and understanding the action mechanisms of bioactive compounds

The selected potential gene targets were analyzed using the Network analyst, and the PPI network was obtained (Table S1, Appendix A; Figure 4). The network diagram shows the close interactions between genes. Moreover, we have selected the biopotential genes (IRS1, TNF-α, IL6, MAPK3, DPP4, LEPR, GSK3B, PPARα and PIK3CG etc.) in the same PPI network to disclose the action mechanism of potentially bioactive compounds (quercetin and gallic acid) of P. granatum.

Therefore, we constructed a global view of the compound-target-pathway network to better understand the molecular mechanism of P. granatum functions to improve diabetes (Figure 5). The network created in Figure 5 provided an understanding of the complex network relationships in terms of the "one ingredient-multiple target" and "multiple ingredient-multiple targets" strategies to treat diabetes and suggested the existence of strongly complex correlations among polyphenols (quercetin and gallic acid) and target genes.

Furthermore, by interacting with α-amylase and α-glucosidase, polyphenols improved oxo-inflammations, dyslipidemia, and reduced carbohydrate digestion and absorption. It is also reported that these compounds controlled insulin levels in the pancreatic beta-cells by enhancing the 5′ adenosine monophosphate-activated protein kinase pathway (Aryaeian et al., 2017; Guasch-Ferré et al., 2017). Furthermore, based on the findings, it is predicted that polyphenolic compounds from P. granatum may control oxo-inflammations, insulin resistance, and glucose metabolism, which are beneficial for the management of diabetes.

Growing evidence suggests that polyphenol consumption has a long history of use in the management of various diseases and disorders including diabetes. Hence, by utilising a network pharmacology and experimental studies, polyphenolic compounds of P. granatum were used to illustrate the understanding of drug action across multiple layers of information. Due to the composite nature of plants, it is an excellent approach for future drug development and design to characterise the pharmacological mechanism of bioactive compounds.
compounds by targeting proteins involved in multiple pathways associated with diabetes and related disorders (Chandran et al., 2017; W. Li et al., 2017).

Quercetin increases liver glucose uptake, decreases hyperglycemia-stimulating GLUT4 and glucokinase, and activates the insulin-independent adenosine monophosphate-activated protein kinase (AMPK) signalling pathway in muscle cells. It also inhibits gluconeogenesis and glycogenolysis in the liver (Babu et al., 2013). In addition, it lowers lipid peroxidation, and reduced glucose absorption by inhibiting glucose transporter 2 (GLUT2) and inhibiting phosphoinositide 3-kinases (PI3K) that are activated by insulin. The oxo-inflammatory, metabolic status, and beta-cell function are improved by quercetin and sitagliptin co-treatment (Al-Ishaq et al., 2019). Furthermore, quercetin regulates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, lowering levels of glycaemia and insulin resistance (Ibrahim et al., 2022).

The oxo-inflammatory, metabolic status, and beta-cell function are directly involved to manage diabetes. Interestingly, the results of this study provide a direction to explore experimental studies to unveil the molecular mechanism of P. granatum for the management of diabetes and related disorders.

CONFLICTS OF INTEREST

The authors declared no conflict of interest.

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A. APPENDIX. SUPPLEMENTARY INFORMATION

Supplementary data to this article can be found online at https://doi.org/10.53365/nrfhh/153385.

AUTHOR CONTRIBUTIONS

MI-Design the study, perform experimental work, analyse data and write the manuscript. RKT provide chemicals, analysed data and interpret, and approved the final manuscript. MF and SN collection and/or assembling of data.

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