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IN VITRO EVALUATION OF PUNICA GRANATUM FRUIT PEEL EXTRACT FOR ITS POTENTIAL ANTI-DIABETIC EFFECTS

Rutuja K More[1]*, Prashant L Pingale[1], Chandrashekhar D Upasani[2], Sunil V Amrutkar[3]

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ABSTRACT
Background: With growing awareness of pomegranate's health benefits, pomegranate products have been consumed more frequently in recent years, and pomegranate peel has emerged as one of the most prevalent wastes in the food industry. Pomegranate Peel concentrate is an indigenous substance with strong antioxidant and antidiabetic actions as a result of its tannins as well as polyphenols content. **Methods:** In the present study, pomegranate skin extract, both liquid and alcohol-based, was evaluated for polyphenolic and flavonoid content. Alcoholic fruit peel extract was also assessed for 1,4-α-D-glucan glucanohydrolase and α-D-glucoside glucohydrolase enzyme activity. **Results:** According to findings, pomegranate peel extract showed significant antioxidant content. Phytochemical analysis of ethanol-derived extract of pomegranate peel found a noteworthy amount of ellagitannins and flavonoids such as Punicalin, Punicalgin, Punicic acid, Catechin, Quercetin, Rutin, and Kaempferol. In contrast, punicalgin, ellagic acid, and gallic acid are responsible for antidiabetic activity. The LC-MS characterization of peel extract of pomegranate showed 10 bioactive compounds. The IC_{50} value for 80% alcoholic extract of pomegranate peel was found to be 5.86 mg/ml of α- amylase and 6.58 mg/ml of α-glucosidase. **Conclusion:** It was found that the inhibition of 1,4-α-D-glucan glucanohydrolase and α-D-glucoside glucohydrolase enzymes could be an effective mechanism by which it can give anti-diabetic effects.

INTRODUCTION

Diabetes mellitus (DM) is an unusually high blood sugar level that causes problems with the manufacturing of insulin, many systemic consequences, and a large contribution to morbidity and mortality in cardiovascular disease. Diabetes is largely categorized into two classes type I and type II. Diabetes, however, can also appear during pregnancy and in other situations, such as when there is pharmacological or chemical toxicity, inherited hormonal disorders, insulin-related problems, or other conditions [1]. Nowadays, diabetes is treated by oral

[1]*Department of Pharmaceutics, GES's sir Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nashik Maharashtra, 422005*
[2]*Department of Pharmacology, SNJB's Shriman Sureshdada Jain College of Pharmacy, Neminagar, Chandwad, Maharashtra, 423101*
[3]*Department of Pharmaceutical Chemistry, GES's sir Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nashik Maharashtra, 422005*

For Correspondence:** **rutujamore16@gmail.com

antidiabetic drugs, which include metformin, chlorpropamide, glimepiride, Pioglitazone, gliptins, and α-glucosidase inhibitors. These synthetic drugs are associated with many complications. It is becoming more apparent that finding a solution for the primary consequences of today's global diabetes epidemic, as well as its complications, which impact equally big and tiny arterial vessels, is also necessary. Most individuals with both kinds of diabetes report experiencing common vascular consequences such as renal disease, lack of vision, and amputations; existing treatments merely slow the course of the disease. Lowering the rate of glomerular filtration, a sign of a failing kidney, is an elevated risk for macrovascular problems like heart attacks and strokes. Numerous different approaches have been investigated in clinical trials for diabetes-related problems, with generally unsatisfactory outcomes [2]. While the precise cause of diabetes is still unknown, experimental data points to the role of free radicals in both the etiology of the disease and, more crucially, in the emergence of its complications [3]. Free radicals can affect biological functioning by destroying lipids, proteins, DNA, and other substances found in cells. Numerous recent studies demonstrate the efficacy of antioxidants in treating clinically generated diabetic conditions in laboratory animals by mitigating free radicals [4,5].

Recent developments and the role that oxidative stress plays in exacerbating diabetes mellitus have prompted efforts to identify appropriate anti-diabetic and antioxidant therapies. Bioactives of natural origin, which are more affordable and safer than these medications, might be used in their place. There is a renewed interest in using herbal remedies to manage diabetes. *Punica granatum* Linn. (Family Lythraceae previously Punicacae), also known as Pomegranate, is a small tree or deciduous shrub that bears fruit and reaches a height of five and eight meters. There are various structural divisions within the pomegranate, from root to fruit. Each of these divisions has unique therapeutic and cytotoxic properties. Different types of chemical constituents obtained from various parts of the plant are observed to elicit varying pharmacological activities, leading to the treatment of several disorders [6,7]. Pomegranate juice constitutes actives like mineral elements and amino acids, while seeds are rich in ellagic acid, fatty acids, conjugated linolic acid, and oleic acid. Many researchers reported that parts of pomegranate, mainly arils and peel, are rich in many bioactive components such as phenolics, flavonoids, ellagitannins, and proanthocyanidins, which all have strong antioxidant properties along with antibacterial and antifungal activities [8,9].

Among the varieties like Ganesh, Arakhta, Bedana, Dholka, and Alandi, grown all over India, the variety Ganesh is more popular because of its prolific yield, medium-sized fruits, soft seeds, pinkish flesh, sweet juice, and consumer acceptance. In the present study, a variety of Ganesh was used as a source of pomegranate fruit peel for experimental purposes. As pomegranate peel contains many anti-oxidants, people don't normally use it. It is thrown away as waste. The proposed study aimed to analyze the phytochemical makeup and possible antioxidant potential of *Punica granatum* skin extracts in both aqueous and ethanolic forms.

MATERIALS AND METHODS

Materials

Fruits of *Punica granatum* were obtained from the local market (Maharashtra, India). These fruits are peeled by hand. Folin-Ciocalteu's phenol reagent, 2,2-diphenylpicrylhydrazyl (DPPH), Acarbose, Gallic acid, Rutin. Analytical grade reagents and solvents were employed.

Making Pomegranate Skin Extract

Gathered pomegranate skin was allowed to air dry in the shade. Dried peels were ground to powder. Two 2-liter Erlenmeyer vessels were used to macerate 100 grams of pulverized *Punica granatum* peel in solvent water and 80% ethanol separately for 48 hr. After the extraction was completed, the micelle was separated from the marc by filtration and concentrated in a water bath. The concentrated extract was dehydrated at 40°C in an oven and kept in a cold location for additional examination [10,11].

Another 100-gm peel powder is extracted with ethanol solvent using a Soxhlet extractor for 16 hr. At the end of the extraction, the ethanol solvent is evaporated to obtain an ethanol crude extract. The extract is stored in a cool and dry place for further analysis [12, 13]. The % Yield of both extracts was carried out.

Phytochemical Evaluation of Ethanolic Extract

The organoleptic evaluation identified physical features by describing the taste, look, color, and odor. The extract's solubility was determined using different solvents, such as Ethyl alcohol, 0.1N hydrochloric acid, 0.1N sodium hydroxide, and

Phosphate buffer solutions (pH 4, 7, and 9). Preliminary phytochemical screening for flavonoids and tannins was done.

A. Tests for flavonoids:

Shinoda Test: Two ml of dilute HCl and a shard of magnesium were mixed into one ml of extract, generating an intense pink color that suggests flavonoids are present.

Alkaline reagent test: Add excess sodium hydroxide until the extract shows deep yellow coloration, which decolorizes after the addition of acid, showcasing the availability of flavonoids.

Lead acetate test: Add 2ml of freshly prepared lead acetate solution to the test solution, resulting in a yellow precipitate showing flavonoids.

B. Tests for Tannins

Ferric chloride test: Five percent 1 or 2 ml $FeCl_3$ solution was added to one ml of extract solution. The solution's blue-green coloration reveals the presence of tannins.

Gelatin test: 2ml NaCl (10%) and a 1% watery gelatin solution are incorporated into a 2ml extract. The precipitate's white, buff appearance suggests the presence of tannins [14,15].

Determination of total phenolic content

Ten milliliters of methanol and water (4:6 v/v) were used to dissolve five milligrams of each dehydrated pomegranate peel extract. A 0.2 ml solution was mixed with 1.0 ml of a Folin-Ciocalteu reagent that had been reduced ten times and 0.8 ml of a 7.5% Na_2CO_3 solution. Using a UV-visible spectrophotometer, the absorption intensity was determined at 765 nm after standing for half an hour at normal temperature. The results were reported as comparable to gallic acid [16].

Estimation of total flavonoid compounds

Flavonoid estimation was done using $AlCl_3$ colorimetric methodology. 1 ml extract was dissolved in 5 ml distilled water. After five minutes, a five percent sodium nitrite solution and a 10% AlCl3 solution were added. Then, 5 ml of 1M of sodium hydroxide was combined into the solution. 4 cc of purified water was added to the mixture to dilute it. Findings of absorbance measurements at 415 nm were expressed as equivalent to rutin [17].

Metabolite screening

Metabolite profiling of the herbal extracts confirms the presence of bioactive compounds according to the presence of their functional moieties [18]. Ethanolic extract was analyzed on an LCUVMS System (Agilent Technologies) equipped with Agilent 6550 Q-TOF series. Mass range (m/z) was maintained in 50-1700. Analysis was done in negative mode. The mass spectra were obtained using a negative polarity mode-operated electromagnetic ionization source. Using an MS scan mode, full-scan data gathering was carried out from m/z 50-1700. The injection volume was 8 µL.

Assessment of Antidiabetic Inhibition

1,4-α-D-glucan glucanohydrolase assay

The extract's enzyme inhibitory activity was tested using a modified version of the usual procedure [19]. A reaction mixture comprising fifty milliliters of phosphate buffer (pH = 6.8), ten microliters of α-amylase, and 20 microliters of extract and control at different doses (1,2,4,8,12,16, and 20 mg/ml) was previously incubated for 20 minutes at 37°C in a 96-well plate. Subsequently, the addition of 100 µl of the dinitrosalicylic acid color agent was done and boiled for 10 minutes, after addition of 20 µl of 1% soluble starch (100 mM phosphate buffer pH 6.8) was added as a substrate and further incubated for 30 minutes at 37°C. Using a Multiplate Reader, the absorbance of the resultant combination was examined at 492 nm. Acarbose was employed as a control at different values that ranged from 1.0 to 20 mg/ml. The equation was used to compute the % inhibition, which was used to express the results.

$$Inhibitory\ activity\ (\%) = \frac{A_c - A_t}{A_c} \times 100$$

Where A_c=Absorbance of Control; A_t=Absorbance of Test

α-glucosidase inhibition assay

The extract's α-glucosidase inhibitory activity was tested using a modified version of the usual protocol [20]. A test mixture comprising 50 microliters of phosphate buffer (pH = 6. 8), 10 microliters of alpha-glucosidase (1 U/ml), and 20 µl of extract and control at different doses (1,2,4,8,12,16, and 20 mg/ml) was preincubated for 15 minutes at 37°C in a 96-well plate. The substrate, 5 mM, 20 microliters P-nitrophenyl glucopyranoside (P-NPG) was then added, and the mixture was left to incubate for 20 minutes at 37°C. Adding 50 µl sodium carbonate (0.1 M) terminated the process. Using a Multiplate Reader, the absorbance of the liberated p-nitrophenol was estimated at 405 nm. Acarbose was used as a control at different values that range from 1 to 20 mg/ml. The equation was used to compute the % inhibition, which was used to express the results.

$$Inhibitory\ activity\ (\%) = \frac{A_c - A_t}{A_c} \times 100$$

Where A_c=Absorbance of Control; A_t=Absorbance of Test

RESULTS AND DISCUSSION

Phytochemical Evaluation of Extract

Table 1 describes the extraction yield of both aqueous and alcoholic extracts obtained from maceration and the soxhlet technique. The soxhlet extraction technique's extract was higher than the extract from maceration, so it was used for further analysis.

Table 1: Extraction yield obtained from Maceration and Soxhlet extraction Techniques

% Yield from Maceration Technique	
Water -15%	Ethanol - 18%
% Yield from Soxhlet Extraction Technique	
Ethanol – 27%	

The dried ethanolic extract was brown with a woody odor and bitter taste. The extract was soluble in tested solvents 95% ethanol, 0.1 N hydrochloric acid, 0.1 N sodium hydroxide, 0.9% sodium chloride, and phosphate buffer solutions (pH 4, 7 & 10). Moreover, Table 2 summarizes the existence of plant components in the extracts. Shinoda tests confirm the presence of flavones and flavonols. Decolorizing the yellow-colored alkali extract mixture indicates the presence of flavones and flavonones. The formation of a jelly precipitate in the gelatin test showed that tannins are present. In contrast, a blue-black color in the ferric chloride test suggests the existence of hydrolyzable tannins, but a brownish-green hue could indicate the presence of compressed tannins. The extract's tannins give it its unpleasant taste and smell of wood.

Table 2: Phytochemical Analysis of Extract

	Test	**Inference**
Flavonoids	Shinoda Test	++
	Alkaline reagent test	++
	Lead acetate test	++
Tannins	Ferric chloride test	++
	Gelatin test	++

Numerous writers have documented flavonoids' various biological activities, including antioxidant, anti-inflammatory, antibacterial, anti-angiogenic, anticancer, and anti-allergic properties previously. Many phenolic compounds, including tannins, act as free radical scavengers [21].

Total phenolic and flavonoid content

The polyphenol concentration of ethanolic extract of pomegranate peel was determined using the reported method. On a commercial basis, solvents like methanol, ethanol, acetone, chloroform, and ethyl acetate are used to extract phenolic chemicals from peel. When extracting antioxidants, polar solvents are more effective than non-polar ones. It has been found that phenolic content ratios and related antioxidant activity vary when extracting solvents differ. In this study, the phenolic from the ethanolic extract was discovered to be 246 ± 2.01 mg GAE/g, comparable to reported values. However, it should be remembered that the extraction solvent utilized can impact variations in the pomegranate's overall phenolic content [22, 23]. The total flavonoid content in ethanolic extract was 54 ± 1.96 mg rutin/g. The study results indicated that phenolics and flavonoids were found in higher quantities in pomegranate peels. The total phenolics in pomegranate peel were found to have antioxidant properties.

Metabolite screening

The LCUVMS analysis of bioactives in the ethanolic extract of pomegranate peel was performed. The method identifies and quantifies the 10 bioactives (polyphenols, flavonoids, and tannins) present in the extract. The chromatographic spectra of the sample are shown in Figure 1. The available research and experiment results were compared to provide a trustworthy characterization. Based on their UV spectra, the chromatographic peaks were preliminarily categorized into a chemical class. UV detectors are commonly employed for routine identification and quantification study of these chemicals, and they offer important details for recognizing bioactive [24]. The quantification data observed from the analysis is listed in Table 3. Samples eluted at 2.81 and 2.84 show molecular ions at 637.0099, and samples eluted at 781.0537 show the presence of gallagic acid and punicalin. Ellagitannins, mainly punicalgin, were identified by molecular ions at 1083.05 with a retention time of 3.09 min. Chemotaxonomical considerations with the LC/UV and LC/MS data made it possible to discover significant polyphenols listed in Table 3 responsible for the extract's antioxidant and antidiabetic activity. Punicalgin is a primary polyphenol found in pomegranate peel that helps to regulate glucose release. Also, in the study on diabetic nephropathy, the findings demonstrate that following punicalagin intervention, diabetic mice's blood urea nitrogen (BUN), serum creatinine (CREA), and urine albumin to creatinine ratio (UACR) all significantly decreased. Additionally, the symptoms of glomerular interstitial hyperplasia and glomerular hypertrophy were lessened [25].

Evaluation of Antidiabetic Activity

Antidiabetic enzyme inhibition was determined by using acarbose as a control. In the present study, a linear concentration range of extract was used. The results were represented by IC_{50} values, presenting the content of the extract that causes 50% blocking of α- α-amylase and α-glucosidase activity. The IC_{50} value for 80% alcoholic extract of pomegranate peel was found to be 5.86 mg/ml of α- amylase and 6.58 mg/ml of α-glucosidase. The concentration of the extract observed linear inhibition. Ellagitannins, i.e., punicalgins, punicalin, and phenolic acids like gallic acid and ellagic acid, may control the inhibitory effect against alpha-glucosidase and α-amylase.

This impact was more noticeable when 20 mg/kg of gallic acid was given instead of 10 mg/kg. According to previous literature, pomegranate flower extract high in gallic acid reduces blood sugar levels via increasing glucose receptor sensitivity [26].

In another study on diabetic rats, ellagic acid also demonstrated hypoglycemic action; 100 mg per kilogram was more efficacious than 50 mg per kilogram. Phytoconstituents like ellagitannins found in peels of pomegranate are punicalgin and punicalin. Studies reported peel extract could decrease blood sugar through regeneration of beta cells [27].

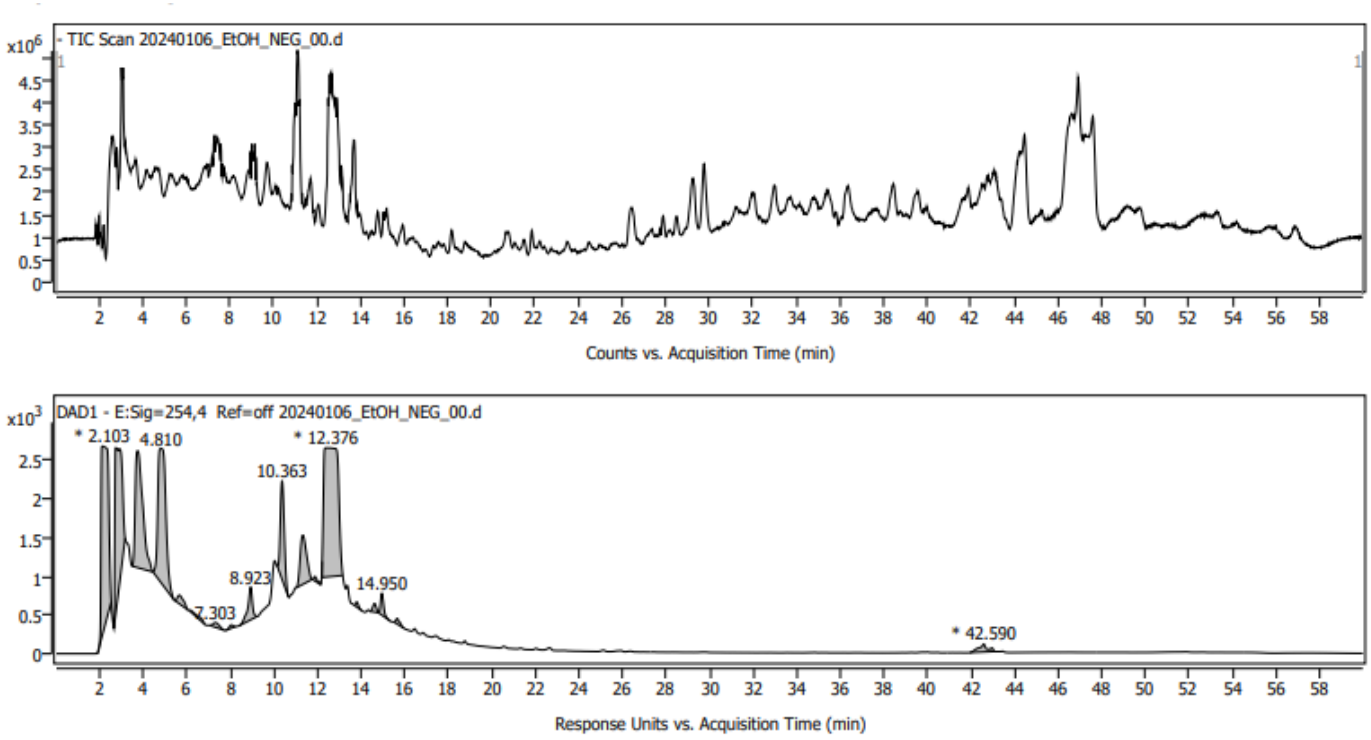

Figure 1: Chromatogram of Pomegranate peel ethanolic extract

Table 3: Retention time (Rt), characteristic ion, Target formula of bioactive in Pomegranate extract

Compound	Bioactive	Retention time (min.)	[M–H]- m/z	Target formula
S1	Gallagic acid	2.811	637.0099	$C_{28}H_{14}O_{18}$
S2	Punicalin	2.844	781.0537	$C_{34}H_{22}O_{22}$
S3	Gallic acid	3.077	169.0143	$C_7H_6O_5$
S4	Punicalgin	3.094	1083.0598	$C_{48}H_{28}O_{30}$
S5	Rutin	12.849	609.1455	$C_{27}H_{30}O_{16}$
S6	Ellagic acid	13.931	300.9986	$C_{14}H_6O_8$
S7	Catechin	15.912	289.0711	$C_{15}H_{14}O_6$
S8	Quercetin	18.192	301.0351	$C_{15}H_{10}O_7$
S9	Kaempferol	20.706	285.0403	$C_{15}H_{10}O_6$
S10	Punicic acid	42.496	277.2165	$C_{18}H_{30}O_2$

Table 4 % Inhibition of enzymes

	1,4-α-D-glucan glucanohydrolase assay		α-glucosidase inhibition assay	
	Peel Extract	**Acarbose**	**Peel Extract**	**Acarbose**
Control	0	0	0	0
1mg	20.90	44.75	2.49	11.97
2mg	26.10	64.77	20.19	30.9
4mg	36.18	80.04	25.78	37.94
8mg	44.18	89.91	32.56	52.56
12mg	51.68	93.63	42.51	69.88
16mg	59.23	95.21	55.91	78.05
20mg	66.02	96.31	64.02	86.08

CONCLUSION

The present study extracted ethanolic extract from the pomegranate peel using the soxhlet and maceration techniques. Ethanolic extract showed a greater yield than aqueous extract. The pomegranate peel's antioxidant qualities are attributed to flavonoids and tannins, which were found in the ethanolic extract according to experimental phytochemical analysis. It was discovered that the ethanolic extract had a higher total phenolic and flavonoid content to exert an antioxidant effect. The preliminary studies revealed that the pomegranate peel has a high phenolic content, which is to be expected given its richness in ellagitannins or phenolic compounds. However, the metabolite profiling of the bioactives in an extract confirmed that pomegranate peel extract is rich in polyphenol content. In-vitro antidiabetic study of ethanolic extract showed significant potential for further research. In vivo, an analysis should evaluate the antidiabetic activity of the hydroalcoholic bioactive pomegranate peel extract. In vivo studies will help show how bioactives stimulate insulin secretion or regulate blood glucose levels.

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NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Prashant L. Pingale reviewed and analyzed the data related to the experiment's design. Rutuja K. More carried out the laboratory experiments and recorded the findings. Chandrashekhar D. Upasani and Sunil V. Amrutkar supervised the study and contributed to the final draft. All authors read and approved the final manuscript.

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