Antioxidant and Antidiabetic Properties of Pectin Extracted from Pomegranate (Punica granatum) Peel

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INTRODUCTION

Diabetes mellitus is a chronic disease resulting from defects in insulin secretion, insulin action or both. The prevalence of diabetes has been increasing gradually over the last few decades. It has emerged as a global health concern that burdens public health and socioeconomic development. Oxidative stress plays a crucial role in developing diabetic complications affecting the eyes, kidneys, nerves, and heart. There are various established adverse effects of current conventional antidiabetic drugs. Due to this fact, plants have been chosen for developing new drugs as the bioactive compounds in plants have higher antidiabetic and antioxidant properties with low toxicity. Pomegranate peel is an agro-industrial waste that is rich in pectin. Pectin is a natural polysaccharide that has gained importance due to its pharmacological properties. In this study, pectin from pomegranate peel was extracted using ultrasound-assisted extraction to characterize the pectin and to evaluate its antioxidant and antidiabetic activity. Pomegranate peel pectin (PPP) was extracted using citric acid (solid-to-liquid ratio of 1:30 g/mL) at pH 2 under optimized conditions at 75°C for 40 min. A white sponge-like solid was obtained upon freeze-drying with a yield of 18.17%. The moisture content of pectin was 18.26% and it was found to be a low-methoxylated pectin with a degree of esterification (DE) of 31.07%. Fourier Transform Infrared Spectroscopy (FTIR) results showed that pectin from pomegranate peel was quite similar to commercial pectin. The antioxidant activity was evaluated using scavenging assays, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 1 mg/mL (78.26% with IC₅₀ value of 326.8 μg/mL, 79.12% with IC₅₀ value of 83.63 μg/mL and 442.7 mg Fe²⁺/g, respectively). The antidiabetic activity was assessed using an α-amylase inhibition assay at 1 mg/mL (43.82%). These results suggest that PPP has the potential to be used as an antidiabetic and antioxidant agents.
side effects and are unable to slow down the progression of diabetic complications. Drugs with low toxicity are in high demand because excessive use of chemical drugs can be detrimental to health. Medicinal plants are the major source of bioactive compounds with therapeutic benefits and for the discovery of new drugs. Many of the bioactive compounds are therapeutic in managing and chronic diseases like diabetes, obesity, cancer, and cardiovascular disease [5]. Foods such as legumes, beans, fruits, and plants contain various phytonutrients. Consumption of phenolic-rich fruits and vegetables has been associated with a lower risk of chronic disease development due to reduced oxidative stress and prevention of macromolecular oxidation [6].

Pomegranate, also known as *Punica granatum* is a drought-tolerant plant with a long life span [7]. Pomegranates can treat several diseases like high blood pressure, high cholesterol, oxidative stress, diabetes mellitus, and inflammatory activities [7]. Plant parts such as fruit peels, seeds, fruit shells and others are often treated as agro-industrial waste and contain active compounds that can help to cure various diseases. If these fruit wastes are not utilized properly, they can end up in landfills which will eventually lead to environmental issues. Pomegranate peel (PP) waste is abundant in pectin [8]. According to recent research, PP is a valuable waste with intriguing pharmacological activities due to its high concentration of phenolics, polysaccharides, and bio-transformed metabolites like urolithins [9]. Pectin is a natural polysaccharide that can be found in plant cell walls. It is widely used in food industries, cosmetics, and pharmaceutical industries. Thus, this study aimed to evaluate the antioxidant and antidiabetic properties of pectin extracted from the peel of *Punica granatum*, which could potentially be used in managing diabetes mellitus.

**METHODOLOGY**

**Sample Preparation**

*Punica Granatum* fruits were obtained from the local market. Then, the fruits were peeled and rinsed with distilled water to remove dirt. Then, the fruit peel was cut into smaller pieces of the same size.

**Preparation of pectin**

**Drying of pomegranate fruit peel**

The fruit peels were dried in an oven at 40°C overnight until a constant weight was achieved. Then, the dried fruit peel was ground into a powder using a dry blender before being stored in a desiccator at room temperature until further use.

**Ultrasound-assisted extraction**

The ultrasound-assisted extraction was conducted according to Chua et al. [10] with slight modifications. 60 g of pomegranate fruit peel powder was dissolved in 0.1 M citric acid at pH 2.0 and 330 W and frequency at 37 kHz during the extraction process. The mixture was allowed to cool before filtering it through Whatman No. 1 filter paper. The filtrate was precipitated with 95% ethanol at a 2:1 (v/v) ethanol-to-filtrate ratio and then allowed to stand overnight (± 15 h) at room temperature (± 25°C). The precipitate was filtered and washed three times using ethanol 96%. The extracted pectin was dried in a freeze-dryer for three days. The equation below was used to calculate pectin yield:

\[
\text{Pectin yield (\%) } = \frac{(\text{Weight of dried pectin (g)})}{(\text{Weight of dried powdered peels (g)})} \times 100\%
\]

**Characterization of pectin**

**Moisture content**

Moisture content was determined using moisture determination balance and it was done at the Institute of Tropical Forestry and Forest Product (INTROP), UPM.

**Degree of esterification (DE)**

The degree of esterification was performed according to Chua et al. [10]. 0.1 g dried pectin was dissolved in 10 mL distilled water after being wet with 1 mL ethanol. The mixture was swirled continuously to ensure thorough pectin dissolution. Then, 3 drops of phenolphthalein indicator were added to the mixture. The solution was titrated with 0.1 M NaOH, and the volume (V1) needed to turn into a pale pink colour was recorded. 5 mL of 0.1 M NaOH was then added to the mixture. At room temperature, the mixture was stirred for 1 h. The mixture must be swirled with 5 mL of HCl until the pink colour fades and then another 3 drops of phenolphthalein were added to the mixture. The solution was titrated with 0.1 M NaOH, and the volume (V2) needed to turn into a pale pink colour was recorded. Using the equation below, calculate the degree of esterification:

\[
\text{DE (\%)} = \frac{V_2}{V_1 + V_2} \times 100\%
\]

Where,

V1: initial titration volume
V2: final titration volume

**Structural analysis**

The functional groups of the pectin extracted from the pomegranate fruit peels were analysed using an FTIR Spectrometer Spectrum which was done at INTROP. The frequency range used was 4000-400 cm\(^{-1}\) [11]. It was then compared with the standard pectin.

**Determination of antioxidant activity**

**2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay**

This assay was conducted based on the method of [12]. 20 μL of 1 mg/mL pectin was mixed with 180 μL of 0.1 mM DPPH in 100% methanol in a 96-well plate. Then, the plate was incubated for 30 min in a dark room. The absorbance reading was recorded using a microplate reader at 540 nm. Different concentrations of samples and ascorbic acid were prepared. The equation below was used to calculate the scavenging activity:

\[
\text{Scavenging activity (\%)} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100\%
\]

Where,

Ac = Absorbance of control
As = Absorbance of sample

**Ferric Reducing Antioxidant Power (FRAP) assay**

This assay was conducted based on [12]. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl\(_3\).6H\(_2\)O in the volume ratio 10:1:1. Then, 20 μL of extracted pectin was mixed with 1 mg/mL with 180 μL of FRAP reagent in a 96-well plate. The plate was incubated for 30 min in a dark room.
The absorbance reading was recorded using a microplate reader at 593 nm. Different concentrations of ferrous sulphate (FeSO₄·7H₂O) as a standard and samples were prepared. The results were expressed as FeSO₄ equivalent mg/g extract.

2. 2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acids) (ABTS) assay

This assay was conducted based on [12]. The ABTS dye was prepared by mixing 5 mL of ABTS solution (7 mmol/L) with 88 μL of potassium persulfate solution (140 mM). Then, the mixture was incubated for 16 h in the dark at room temperature. The prepared ABTS solution was diluted with analytical grade ethanol to obtain an initial absorbance of 0.7 at 734 nm. 10 μL of the sample was added to a 96-well plate along with a 290 μL previously prepared ABTS solution. The mixture was incubated for 6 min in the dark room at room temperature. The absorbance reading was recorded using a microplate reader at 734 nm. Different concentrations of ascorbic acid as standard and samples were prepared. The equation below was used to calculate the scavenging activity:

\[
\text{Scavenging activity} (\%) = \left( \frac{\text{Ac} - \text{As}}{\text{Ac}} \right) \times 100\%
\]

Where,

Ac = Absorbance of control
As = Absorbance of sample

\(\alpha\)-amylase Inhibitory Activity Assay

The inhibition assay was conducted according to [13] with slight modifications. The assay mixture contains 200 μL of 0.02 M sodium phosphate buffer pH 6.9 with addition of 0.006 M sodium chloride solution, 200 μL of α-amylase solution (2 U/mL) and 200 μL of 1 mg/mL of extracted pectin was incubated at 37°C for 15 min. Then, 200 μL of 1% starch solution was added as a substrate and re-incubated at 37°C for 15 min. The reaction was terminated by the addition of 200 μL of 3.5-dinitrosalicylic acid (DNS) reagent. It was then incubated in a boiling water bath at 90°C before adding 5 mL of distilled water. The absorbance reading measured the absorbance at 540 nm using a microplate reader. Negative control was prepared by replacing the sample with distilled water to show 100% enzyme activity, while a blank mixture was prepared without the enzymes. The inhibitory activity of the sample was compared with 1 mg/mL of acarbose as a positive control. The equation below was used to calculate inhibitory activity.

\[
\text{Inhibitory activity} (\%) = \left( \frac{\text{A}_{00} - \text{A}_{10}}{\text{A}_{00}} \right) \times 100\%
\]

Results and Discussions

Extraction of pomegranate peel pectin (PPP)

Ultrasound-assisted extraction (UAE) method was applied in this present study to extract pectin from Punica granatum. Pectin extracted using conventional methods is not only low in quantity but also in quality, as extended exposure to heat during extraction can lead to the degradation of pectin [14]. The extraction was done at 70°C for 40 min to ensure promising extraction quality. [15] reported that temperatures ranging from 70 to 90°C can give higher pectin yield. Hence, 70°C was chosen since higher temperatures would cause the molecules of pectin composed of α-(1-4) linked units of galacturonic acid or methyl ester to degrade, leading to lower pectin yield. The highest pectin yield was 26.91% obtained at 45 min whereas the lowest pectin yield was 9.34% obtained at 30 min. It has been shown previously that raising the extraction temperature and time within a given range results in higher pectin yield, whereas decreasing or raising the temperature and time above a particular level results in low pectin production [16].

The extracted pectin was subjected to freeze-drying for further analysis. Freeze-drying did not influence pectin content [17]. A sponge-like solid was obtained upon freeze drying and it dissolved very quickly in distilled water due to its soft texture. The texture appeared to be soft and can be crushed easily [18]. In this study, the pectin yield obtained was 18.17% under the optimized conditions. Previous studies reported that pectin yields were 6.8 to 10.1% [19], 23.87% [20] and 8.5% [21]. It can be deduced that pomegranate peels are a good source for pectin production.

Characterisation of pectin

The degree of esterification is commonly used to characterise pectin since it determines its gelling properties in industrial applications. Pectin is classified as low methoxyl pectin (DE >50%) or high methoxyl pectin (DE ≥50%) [10]. The titrimetric approach was used to determine the degree of esterification of pectin. In this study, the degree of esterification of pectin obtained from pomegranate peel was 31.07%. In agreement with this, several studies reported that the degree of esterification of pectin extracted from pomegranate peel ranged from 30-38% [11,19,22]. The pectin obtained from Punica granatum peel was categorised as LM pectin since the percentage DE was less than 50%. Low-methoxylated pectin can form gels with or without sugar in the presence of divalent cations. It is extensively utilised in the production of low-sugar foods such as jams and jellies [23].

Moisture content indicates the purity of pectin, and it was determined using moisture determination balance. The moisture content of extracted pectin was 18.26%. Pectin should have a low moisture content for safe storage and to inhibit microbial growth. Due to the formation of the pectinase enzyme, products with a high moisture content may lose quality [24]. In the previous studies, it was found that the moisture content of pomegranate peel pectin was 3.959% and 10.31%, respectively [25,26].

Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out to confirm the functional group of pectin from pomegranate peel and standard pectin. An absorbance spectrum graph is produced by an FTIR spectrometer. It relies on infrared light to scan samples and examine bond properties. Various functional groups will absorb infrared radiation at different wavelengths. A catalogued spectra for known materials can be used to determine the unidentified sample. Fig. 1 describes that extracted pectin and standard pectin had quite similar FTIR spectrums. Pectin is a polysaccharide that is in abundant carboxyl and hydroxyl functional groups [27]. The major peaks of hydroxyl groups appeared at 3305 cm⁻¹ for pectin and standard pectin whereas the major peaks for carboxyl groups were shown at 3300 cm⁻¹.
Antioxidant activity

DPPH assay
The antioxidant activity of pomegranate peel pectin was measured by using DPPH, FRAP and ABTS assays. In the DPPH assay, the extract reduces a violet-coloured DPPH solution to a yellow-coloured product, diphenylpicryl hydrazine. It is extensively used as it can be done within a short period of time [28]. Fig. 2 shows the scavenging activity of PPP with ascorbic acid as the standard. The scavenging activity of pectin at 1 mg/mL concentration was 78.26%.

Fig. 2. The scavenging activity (%) of PPP and ascorbic acid as a positive control at a 1 mg/mL concentration. Data were expressed as mean ± SD (n = 3). **** indicates a high significant difference at p < 0.05 of unpaired t-test obtained from GraphPad Prism software. Error bars represent standard deviation.

The data are presented in IC50 values which represent the concentration needed to inhibit 50% of DPPH. Fig. 3 shows the IC50 values of PPP and ascorbic acid acts as the positive control. Distilled water was used as the solvent. Based on the figure, ascorbic acid showed a significantly lower IC50 value of 149.5 ± 37.43 μg/mL compared to pectin.

Fig. 3. The IC50 value (μg/mL) of PPP and ascorbic acid as a positive control for DPPH scavenging activity. Data were expressed as mean ± SD (n = 3). **** indicates a high significant difference at p < 0.05 of unpaired t-test obtained from GraphPad Prism software. Error bars represent standard deviation.

ABTS assay
The ABTS assay is regarded as one of the most sensitive assays to measure antioxidant activity because the antioxidant reaction has a faster reaction kinetics [29]. The ABTS is initially subjected to an oxidation reaction with potassium permanganate, potassium persulfate or 2, 2’-azo-bis (2-amidinopropane), producing the radical cation of the ABTS (ABTS•+) with a blue-greenish colour that absorbs at wavelengths of 415, 645, 734, and 815 nm. Fig. 4 shows the scavenging activity of PPP with ascorbic acid as the standard. The scavenging activity of pectin was 79.12% at 1 mg/mL. This shows that the scavenging activity of pectin is in close proximity to the values obtained in DPPH and ABTS assay.

Fig. 4. The scavenging activity (%) of PPP and ascorbic acid as a positive control at the highest concentration (1 mg/mL). Data were expressed as mean ± SD (n = 3). **** indicates a high significant difference at p < 0.05 of unpaired t-test.

The data are presented in IC50 values which represent the concentration needed to inhibit 50% of DPPH. Fig. 5 shows the IC50 values of PPP and ascorbic acid as the positive control. The IC50 value of pectin was 83.63 ± 26.02 μg/mL which is slightly higher than ascorbic acid. It was suggested that the presence of hydroxyl groups in polysaccharides such as pectin might be responsible for its high antioxidant activity [30].
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