COMPARATIVE EVALUATION OF ANTIOXIDANT AND ANTIDIABETIC POTENTIALS OF AQUEOUS SEED AND FRUIT EXTRACTS OF PUNICA GRANATUM

Running title: Antioxidants and antidiabetic properties of aqueous seed and fruit extract of Punica granatum.

Type of study: Original research

Padmapriya.A
Department of Biochemistry
Saveetha Dental College and Hospitals
Saveetha Institute of Medical and Technical Sciences
Saveetha University, Chennai-600 077, India
E-mail: padmapriya11102@gmail.com

V. Vishnu Priya
Professor
Department of Biochemistry
Saveetha Dental College and Hospitals
Saveetha Institute of Medical and Technical Sciences
Saveetha University, Chennai-600 077, India
E-mail: vishnupriya@saveetha.com

Kavitha.S
Lecturer
Department of Biochemistry
Saveetha Dental College and Hospitals
Saveetha Institute of Medical and Technical Sciences
Saveetha University, Chennai-600 077, India
Email: kavithas.sdc@saveetha.com

R. Gayathri
Associate Professor
Department of Biochemistry
Saveetha Dental College and Hospitals
Saveetha Institute of Medical and Technical Sciences
Saveetha University, Chennai-600 077, India
E-mail: gayathri.sdc@saveetha.com

Selvaraj. J
Associate Professor
Department of Biochemistry
Saveetha Dental College and Hospitals
Saveetha Institute of Medical and Technical Sciences
Saveetha University, Chennai-600 077, India
E-mail: selvarajj.sdc@saveetha.com

Corresponding author*: Dr R Gayathri
Associate Professor,
Department of Biochemistry,
Saveetha Dental College and Hospitals,
Saveetha Institute of Medical and Technical Sciences,
Saveetha University, Chennai - 600077
ABSTRACT:
Aim: The aim of the study is to compare the antioxidant and antidiabetic activity of ethanolic fruit and seed extract of *Punica granatum*.

Introduction: Diabetes mellitus, Also known as diabetes, is a group of metabolic disorders characterised by high glucose levels which causes hypoglycemia ie., type-2 diabetes, it also causes an increase in level of sugar in blood. Diabetes is a genuine complex and also the fastest growing chronic disease since the 21st century. Increasing the production of insulin causes hyperinsulinemia. (1)*Punica granatum* consists of various pharmacological and biological activities. *Punica granatum* is also called pomegranate which belongs to the family Lythraceae. The metabolites of *Punica granatum* have various types of glucose, fatty acids, vitamins, polyphenols etc. *Punica granatum* also has an ability to prevent diseases like lung cancer, anaemia(2), myocardial infarction, infertility etc. *Punica granatum* is one of the major plants which is rich in phytochemicals, which directly contributes to antioxidant activity.(2)

Materials and methods: The ethanolic seed and fruit extracts of *Punica granatum* are prepared and in vitro antidiabetic effect was studied by alpha-amylase and alpha-glucosidase inhibitory activity. The data were subjected to statistical analysis using one – way analysis of variance (ANOVA) and Duncan’s multiple range test to assess the significance of individual variations between the groups. In Duncan’s test, significance was considered at the level of p<0.05.

Result: The results revealed that both the extracts are rich in phytochemicals and possessed potent in vitro antioxidant activity. Both the seed and fruit extracts possessed alpha amylase and alpha glucosidase inhibitory activity in a concentration dependent manner, although the activities are less than the standard drug, acarbose. This indicates the in vitro antidiabetic activity of the extracts. Among the two extracts, seed extract showed higher activity compared to fruit extract.

Conclusion: The comparative analysis showed that *Punica granatum* seed extract showed more in vitro antioxidant and antidiabetic activity than fruit extract.

Keywords: Diabetes mellitus, phytochemicals, *Punica granatum*, biological activities. Innovative technology, novel method.

INTRODUCTION:
Diabetes mellitus is one of the most endocrine disorders which causes hypoglycemia ie., type-2 diabetes, it also causes an increase in level of sugar in blood. Diabetes is a genuine complex and also the fastest growing chronic disease since the 21st century. Increasing the production of insulin causes hyperinsulinemia (1,3). Drugs like acarbose have inhibitory activity to prevent diabetes but acarbose causes side effects like flatulence and abnormal bloating (4). Therefore there is an urgent need for drugs which are natural in origin and have less side effects. Chemical constituents of medicinal plants are more effective. Alpha-amylase and alpha-glucosidase are the major enzymes which promote digestion of carbohydrates. Alpha amylase breaks polysaccharides into disaccharides whereas alpha-glucosidase breaks disaccharides to monosaccharides that leads to increase in level of sugar in blood (5). Alpha- amylase and alpha-glucosidase are also called potential targets for treating diabetes. Free radicals are a more reactive species which causes oxidative stress therefore results in diabetes, stroke atherosclerosis, cancer and cardiovascular diseases. Dietary antioxidants like ascorbate, carotenoids and tocopherol from natural compounds could reduce oxidative stress (6). Medicinal plants are a natural source which has less side effects and has an ability to scavenge free radicals. Plants have been used for many centuries to prevent diseases, therefore these plants are pharmacologically active. *Punica granatum* consists of various pharmacological and biological activities. *Punica granatum* is also called pomegranate which belongs to the family Lythraceae (2). *Punica granatum* is one of the major plants which is rich in phytochemicals, which directly contributes to antioxidant activity. The colour of *Punica granatum* contains a compound called Anthocyanin. Anthocyanin is a glucose molecule which causes red pigment in *Punica granatum*. It is rich in ascorbic acid and phenolic acids. *Punica granatum* is rich in phytochemicals like gallic acid, flavonoids, amino acids, saponins etc (7). It also exhibits anti allergic, anti inflammatory, antioxidant, vasodilatory properties etc. The seed of *Punica granatum* has an ability to treat skin and breast cancers. Ellagic acid is a main constituent of *Punica granatum* which has high antioxidant properties.(8) The metabolites of *Punica granatum* have various types of glucose, fatty acids, vitamins, polyphenols etc. *Punica granatum* also has an ability to prevent diseases like lung cancer, anaemia, myocardial infarction, infertility, atherosclerosis etc.(9), (10), (11), (12), (13), (14), (15), (16), (17), (18), (19), (20), (21), (22), (23), (24), (25), (26), (27), (28), (29). This present...
study is to compare antioxidants and antidiabetic properties of aqueous seed and fruit extracts of *Punica granatum*. (30).

**MATERIALS AND METHODS:**

**Comparative antioxidant and antidiabetic potentials of aqueous seed and fruit extracts of *Punica granatum***

**1. Phytochemical Screening test**

**Test for phlobatannins**

1 ml of the extract was treated with 1 ml of 1% HCl and boiled for 10 mins. The formation of red color precipitate indicates the presence of phlobatannin.

**Test for Carbohydrates**

Three to five drops of Molisch reagent was added with 1 mL of the extract and then 1 mL of concentrated sulphuric acid was added carefully through the side of the test tube. The mixture was then allowed to stand for two minutes and diluted with 5 mL of distilled water. The development of a red or dull violet ring at the junction of the liquids showed the presence of carbohydrates.

**Test for Flavonoids**

Few drops of 1% liquid ammonia were taken in a test tube and along with it 1 ml of the extract was added resulting in the formation of yellow color thereby indicating the presence of flavonoids.

**Test for Alkaloids**

2 ml of sample was mixed with 2 ml of HCl. Then 6 drops of HCN was added and further 2 drops of picric acid was added that resulted in a creamish pale yellow ppt indicating the presence of alkaloids.

**Test for Terpenoids**

2 ml of sample along with 2 ml of chloroform and 3 ml of conc. H2SO4 was added. Red color ppt obtained indicates the presence of terpenoids.

**Test for proteins**

One milliliter of ninhydrin was dissolved in 1 mL of acetone and then a small amount of extract was added with ninhydrin. The formation of purple colour revealed the presence of protein.

**Detection of saponins**

Foam test: A fraction of the extract was vigorously shaken with water and observed for persistent foam.

**Test for steroids**

One milliliter of chloroform was mixed with 1 ml of extract and then ten drops of acetic anhydride and five drops of concentrated sulphuric acid were added and mixed. The formation of dark red colour or dark pink colour indicates the presence of steroids.

**2. DPPH free radical scavenging activity seed and fruit extracts of *Punica granatum***

Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical was assessed by the method of Hatano et al, (1989). DPPH solution (1.0 ml) was added to 1.0 ml of extract at different concentrations (0.1 to 0.5 mg/ml). The mixture was kept at room temperature for 50 minutes and the activity was measured at 517 nm. Ascorbic acid at the same concentrations was used as standard. The capability to scavenge the DPPH radical was calculated and expressed in percentage (%) using following formula:

\[
\text{DPPH radical scavenging } (\%) = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

**3. In vitro antidiabetic activity**

**3.1. In vitro alpha amylase inhibitory activity of seed and fruit extracts of *Punica granatum***

\(\alpha\)-amylase inhibitory activity of the extract was carried out according to the standard method of Ademiluyi et al [14]. In a test tube, reaction mixture containing 500 microliters of phosphate buffer (100 Mm, pH = 6.8), 100 μl of \(\alpha\)-amylase (2 U/ml) and varying concentrations of T. ammi oil (0.1 to 0.5 mg/ml) was pre incubated at 3 degree Celsius for 20 min. Then, 200 μl of 1% soluble starch (100mM phosphate buffer of pH = 6.8) was added as a substrate and incubated further at 37 degree Celsius for 30min; 1000 microliters of 3,5- dinitrosalicylic acid (DNS) color reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm using multiple readers. Acarbose at various concentrations (0.1-0.5 mg/ml) was used as standard. The results were expressed as percentage inhibition, which was then calculated using the formula,

\[
\text{Inhibitory activity } (\%) = (1 - \frac{A_s}{A_c}) \times 100
\]

Where, \(A_s\) – absorbance in presence of test substance, \(A_c\) – absorbance of control
3.2. α-glucosidase inhibitory activity of seed and fruit extracts of *Punica granatum*

α-glucosidase inhibitory activity of extract was carried out according to the standard method of Ademiluyi et al [14]. In a test tube, reaction mixture containing 500 μl of phosphate buffer (100 Mm, pH = 6.8), 100 microliters of α-glucosidase (1 U/ml) and varying concentrations of T. ammi oil (0.1 to 0.5 mg/ml) was pre incubated at 37°C for 20 min. The reaction was stopped by adding 50 μl of Na2CO3 (0.1 M). The absorbance of the released p-nitrophenol was measured at 405 nm using multiple readers. Acarbose at various concentrations (0.1-0.5mg/ml) was used as a standard. The result was expressed as percentage inhibition which was calculated using the formula,

\[
\text{Inhibitory activity (\%) } = \left(1 - \frac{A_s}{A_c}\right) \times 100
\]

Where,

- \(A_s\) – absorbance in presence of test substance,
- \(A_c\) – absorbance of control

**Statistical Analysis**

The data were subjected to statistical analysis using one – way analysis of variance (ANOVA) and Duncan’s multiple range test to assess the significance of individual variations between the groups. In Duncan’s test, significance was considered at the level of \(p<0.05\).

**RESULTS AND DISCUSSION:**

*Table 1*: qualitative phytochemical analysis of punica granatum seed and fruit extract:

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th><em>Punica granatum</em> seed extract</th>
<th><em>Punica granatum</em> fruit extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Proteins</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>
Figure 1: In vitro antioxidant activity of seed and fruit extracts of *Punica granatum*

![DPPH radical scavenging activity](image1)

Figure 1: Bar graph depicts the In vitro antioxidant activity of aqueous seed and fruit extract of *Punica granatum*. The X axis represents the different concentrations of *Punica granatum* seed and fruit extract taken and the Y axis represents the percentage of inhibition. Blue colour denotes vitamin C, Orange colour denotes seed extract and grey colour denotes fruit extract of *Punica granatum*. The difference was statistically significant. Each line represents Mean ± SEM of 3 independent observations. Significance at $p \leq 0.05$.

Figure 2: In vitro alpha amylase inhibitory activity of seed and fruit extracts of *Punica granatum*

![Alpha amylase inhibitory activity](image2)

Figure 2: Bar graph depicts the In vitro Alpha amylase inhibitory activity of aqueous seed and fruit extract of *Punica granatum*. The X axis represents the different concentrations of *Punica granatum* seed and fruit extract taken and the Y axis represents the percentage of inhibition. Blue colour denotes concentration of standard drug acarbose, red colour denotes seed extract and green colour denotes fruit extract of *Punica granatum*. The
difference was statistically significant. Each line represents Mean ± SEM of 3 independent observations. Significance at p ≤ 0.05.

Figure 3: Bar graph depicts the Alpha glucosidase inhibitory activity of aqueous seed and fruit extract of Punica granatum. The X axis represents the different concentrations of Punica granatum seed and fruit extract taken and the Y axis represents the percentage of inhibition. Yellow colour denotes standard drug acarbose, green colour denotes seed extract and blue colour denotes fruit extract of Punica granatum. The difference was statistically significant. Each line represents Mean ± SEM of 3 independent observations. Significance at p ≤ 0.05.

The results of qualitative phytochemical analysis (table-1) of Punica granatum seed and fruit extracts. Punica granatum seed extract is rich in carbohydrates, saponins, amino acids, proteins, alkaloids and terpenoids. Punica granatum fruit extract is rich in carbohydrates, amino acids, proteins, alkaloids and terpenoids. Phytochemical screening results show that the aqueous seed and fruit extract of Punica granatum is rich in amino acids, flavonoids, alkaloids, terpenoids, and fairly present in saponins and flavonoids. Whereas seed extract is rich in carbohydrates saponins and fairly present in flavonoids. The secondary metabolites contribute significantly towards the biological activities such as antioxidant, antidiabetic, anti-inflammatory, etc. Phytochemicals are mostly rich in greenery which prevents free radical formation. Hence the presence of the phytochemicals in the extracts might have contributed to their beneficial activities.

Figure 3: Bar graph depicts the Alpha glucosidase inhibitory activity of aqueous seed and fruit extract of Punica granatum. The X axis represents the different concentrations of Punica granatum seed and fruit extract taken and the Y axis represents the percentage of inhibition. Yellow colour denotes standard drug acarbose, green colour denotes seed extract and blue colour denotes fruit extract of Punica granatum. The difference was statistically significant. Each line represents Mean ± SEM of 3 independent observations. Significance at p ≤ 0.05.

Aqueous extracts of Punica granatum exhibited in vitro antioxidant activity in a concentration dependent manner. Vitamin-C was used as the standard drug for comparing the antioxidant activity. Free radicals are more reactive species which causes oxidative stress therefore results in diabetes stroke atherosclerosis, cancer and cardiovascular diseases. Dietary antioxidants like ascorbate, carotenoids and tocopherol from natural compounds could reduce oxidative stress. Medicinal plants are a natural source which has less side effects and has an ability to scavenge free radicals. Hence our extracts show potent in vitro antioxidants activity which was evident from the DPPH radical scavenging activity.(figure-2).(32)

Aerosol dependent alpha-amylase activity was observed in both the extracts. Alpha amylase breaks polysaccharides into disaccharides whereas alpha-glucosidase breaks disaccharides to monosaccharides that leads to increase in level of sugar in blood. Alpha- amylase and alpha-glucosidase are also called potential targets for treating diabetes.(34) In the present study the standard drug acarbose showed greater activity compared to the extracts in all the tested concentrations. drugs like acarbose having inhibitory activity on the enzyme possesses inhibition. But these drugs have common side effects such as flatulence and abnormal blotting.(35) Hence there is an urgent need for drugs which are natural in origin and have less side effects. Our study showed the alpha-amylase(figure-2) and alpha-glucosidase(figure-3) activity of Punica granatum, although the activity of standard drugs is more when compared to the plant. Since the extract is natural in origin it may not produce any side effects. Hence the plant extracts are used as antidiabetic agents when further details are done in it.
CONCLUSION:
Thus, from the present study it can be concluded that aqueous seed extract of *Punica granatum* showed more potent in in-vitro antioxidant activity which was evident from the DPPH radical scavenging assay. A dose dependent anti-diabetic activity was observed for the extract and the standard drug acarbose. In the present study the standard drug acarbose showed greater activity compared to the extract in all the tested concentrations.

ACKNOWLEDGEMENT
The authors would like to thank Saveetha Dental College and Hospitals, Saveetha Institute of medical and technical Sciences, Saveetha University for providing research laboratory facilities to carry out the study.

SOURCE OF FUNDING
The present study was supported by the following agencies.
- Saveetha Institute of Medical and Technical Sciences (SIMATS)
- Saveetha Dental College
- Saveetha University
- National insurance building, parrys

STATEMENT OF CONFLICT OF INTEREST
The author declares that there is no conflict of interest in the present study.

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