Phytochemical Analysis and Antioxidant Activity of Wild Pomegranate Collected from Patnitop, Jammu & Kashmir

Ramanjeet Kaur, Lubna Aslam, Nisha Kapoor and Ritu Mahajan*

School of Biotechnology, University of Jammu, Jammu, India.

http://dx.doi.org/10.13005/bbra/2637

(Received: 19 May 2018; accepted: 28 May 2018)

Wild pomegranate is an ancient fruit with known medicinal and curative properties, attributing to overall positive health. The present study focuses on phytochemical analysis and the antioxidant potential of the fruits (red and green), leaves and flowers of wild pomegranate. High amount of carbohydrates and proteins were observed in red fruits, followed by green fruit, while they were low in flowers and leaves. However, leaves possessed higher amounts of phenolics and tannins as compared to other tissues and fruit extracts. Highest radical scavenging effect was observed in red fruit with EC50 value 70.33µg/ml as compared to other tissues. The ferric reducing potential was significantly higher in red fruit (310.99 ± 0.98 µmol Fe²⁺/g dry matter) in comparison to green fruit extracts of wild pomegranate. The results indicated that methanolic extract of red fruits are pharmacologically more active and can be exploited for studying the pharmacokinetics of various bioactive compounds present in wild pomegranate.

Keywords: Wild pomegranate; antioxidants; secondary metabolites; phytochemicals; antioxidants.
MATERIALS AND METHODS

Collection of plant materials
Fruits (green and red), leaves and flowers of wild pomegranate were collected from the Patnitop, Jammu and Kashmir (2,024m above sea level). They were washed with deionised water and disinfected with 0.1% HgCl2 solution for 5 min and then dried in shade. The plant parts were separately grounded to fine powder using an electrical blender, sieved and then stored in vials at 4p C.

Preparation of extracts
Ten grams of powdered material of four samples (flowers, leaves, green fruits and red fruits) were extracted with 20 ml of methanol. After extracting thrice with methanol, the supernatant obtained was air dried using vacuum, in a rotary-evaporator at 40p C. The extracts were then stored at 4p C for further phytochemical analysis.

Phytochemical screening
Phytochemical tests were conducted in four samples (tissues and fruits) with the methanolic extracts using the standard methods.7,8.

Test for carbohydrates
One ml of the each extracts was added to 2 ml of molish reagent (±-napthol in 5% alcohol), followed by the addition of 1 ml concentrated H2SO4. Formation of red or dull violent colour at interphase indicated presence of carbohydrates.

Test for proteins
One ml of methanolic extract of each sample was added to 0.5 ml of 40% sodium hydroxide in a tube. Formation of violet color indicated the presence of proteins.

Test for Flavonoids
One ml each of the extracts was added to 1 ml of 2N sodium hydroxide in a tube. Formation of yellow colour indicated the presence of flavonoids.

Test for Phenols
One ml of each of the methanolic extract was added to 2 ml of 10% ferric chloride in test tube. Formation of blue/green colour indicated the presence of phenols.

Test for Tannins
One ml of the each of methanolic extract was taken in tube to which 1 ml of 5% ferric chloride was added. Formation of dark blue / greenish black colour indicated the presence of tannins.

Test for Terpenoids
One ml of methanolic extract of each sample was added to 2 ml chloroform in test tube followed by addition of concentrated sulphuric acid. Formation of red brown colour at the interface indicated the presence of terpenoids.

Test for Saponins
One ml of each of the extracts was added to 1 ml distilled water in a tube and the mixture was further shaken for 15 min. Formation of 1 cm layer of foam indicated the presence of saponins.

Test for Steroids
One ml of the methanolic extract of each sample was added to 2 ml chloroform followed by the addition of 1 ml sulphuric acid. Formation of reddish brown ring at interface indicated the presence of steroids.

Quantitative determination
Determination of carbohydrates
Total carbohydrate content in methanolic extracts of four samples was calculated using DNS method.9. DNSA reagents and 40% potassium sodium tartarate were freshly prepared. 1 ml DNSA reagent was added to each tube containing different extracts and incubated for 5 min at 90p C, followed by addition of 1 ml potassium sodium tartarate. Absorbance was recorded at 540 nm. Dextrose was used as standard.

Determination of total protein content
Total protein content was estimated using Lowry’s method.10. The reagents prepared were solution A (2% sodium carbonate in 0.1N NaOH) and solution B (0.5% copper sulphate solution in 1% sodium potassium tartarate). Alkaline copper sulphate solution was prepared by mixing solution A and B in the ratio of 50:1. Folin–Ciocalteau (FC) reagent was diluted with equal volume of water just before use. 5ml of copper sulphate solution was added to each extract and incubated at room temperature for 10 min, followed by addition of 0.5 ml FC reagent. Absorbance was recorded at 660nm. BSA was taken as standard.

Determination of total phenolic content
Total phenolics were estimated using Folin-ciocalteau assay.11. 1N folin –ciocalteau reagent was freshly prepared and added to 100 µl of each methanolic extract. The reaction was stopped by adding 1ml of 7.5% sodium carbonate and the mixture was further incubated at room temp for 2 hours. Absorbance was recorded at 760nm.
Table 1. Qualitative analysis of phytochemicals in wild pomegranate extracts

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Phytochemical tests</th>
<th>Green fruit</th>
<th>Red fruit</th>
<th>Flower</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Terpenoids</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Saponins</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Steroids</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Flavonoids</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5.</td>
<td>Coumerins</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Tannins</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>7.</td>
<td>Proteins</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>8.</td>
<td>Carbohydrates</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9.</td>
<td>Phenolics</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

(+++) = present in very high amount  (++)= High amount  (+)= Traces  (-)= Absent

Fig. 1. Analysis of phytochemical content in crude extract of wild pomegranate (L-leaf, F-flower, GF-green fruit and RF-red fruit)
initial DPPH absorption in relation to the control and IC50 value was determined.

**Ferric Reducing Anti-oxidant Power (FRAP assay)**

Antioxidant activity of four methanolic extracts was assessed by FRAP assay. It is based on the principle of reducing ferric to ferrous ions at low pH that results in the formation of a coloured ferrous–tripyridyltriazine complex. FRAP reagents includes 300 mmol/l acetate buffer (pH 3.6), 10mmol/l TPTZ in 40 mmol/l HCl and 20mmol/l FeCl₃. 3 ml of the FRAP reagent was added to 100 µl of each extract and incubated at 37°C for 5 min. Absorbance was taken at 593nm using FeSO₄ as the standard solution.

**Statistical analysis**

All Experiments were conducted in triplicate and results are presented as the mean ± standard Error (SE).

---

**Fig. 2.** Inhibitory concentration of BHT (standard) and extracts of wild pomegranate (L-leaf, F-flower, GF-green fruit and RF-red fruit)

**Fig. 3.** Determination of ferrous reducing capacity of methanolic extracts of wild pomegranate (L-leaf, F-flower, GF-green fruit and RF-red fruit)
RESULTS AND DISCUSSION

Secondary metabolites have pharmaceutical properties that contribute towards human health. Many plants rich in flavonoids, alkaloid and terpenoids have anti-cancerous properties.

Quantitative phytochemical analysis

Total carbohydrate content

A significant amount of carbohydrate content in different parts of wild pomegranate was observed. Maximum carbohydrate content was observed in the red fruit extract (270.58 mg DE/g) followed by green fruit (149.25 mg DE/g), flower (138.58 mg DE/g) and was least in the leaves (137.41 mg DE/g of extract) (Figure 1). This is due to the process of fruit ripening which causes hydrolysis of starch that leads to accumulation of simple sugars in ripe fruit.

These results are in agreement with Zarie et al., where increase in total sugar content from 7.40 mg/100g (20 days) to 17.88 mg/100 g (140 days) was observed during ripening in cultivated pomegranate.

Determination of total phenolic compounds

The total phenolics were estimated using the Folin-Ciocalteau colorimetric reagent. Total phenolic content was highest in leaves (59.67 mg gallic acid equivalent /g of extract), followed by flowers (49.55 mg GAE/g), green fruits (37.90 mg GAE/g) while low phenolics were observed in red fruits (32.86 mg gallic acid equivalents/g) (Figure 1). This decline in the total phenolic level in fruits as compared to tissues is due to the oxidation of phenolic content by polyphenol oxidase that characterizes the stages of maturity.

Determination of tannins

The concentration of tannins was recorded highest in leaves (253.98 mg TAE equivalent /g of extract) as compared to flowers (167.84 mg TAE/ g of extracts) (Figure 1). In fruits, green fruits had more tannins (123.55 mg TAE/g of extracts) as compared to red fruits (95.67 mg TAE / g) as the content of total tannins decreases considerably during ripening and low tannins content also reduces the astringency of red fruits. The decrease in condensed tannin content during ripening process is due to increase in activity of enzymes such as anthocyanin synthase and 3-glycosyl transferase which are involved in formation of anthocyanins.

Determination of proteins

Highest protein content was observed in red fruit (102.83 mg BSA equivalent /g of extract) while it was only 78.05 mg/g in green fruit. Flower and leaves contained 78.03 mg/g and 99.06 mg BSA equivalent / g of extract of proteins respectively (Figure 1). This is due to synthesis of proteins during ripening which was also observed in ripe fruits of cultivated pomegranate.

Determination of antioxidant activity of wild pomegranate extracts

Plants contain many phytonutrients which helps in preventing the damage caused by reactive oxygen species. The antioxidant activity of wild pomegranate extracts was determined by DPPH (2, 2-diphenyl-1-picylhydrazyl) assay and FRAP (Ferric reducing antioxidant activity) assay.

Antioxidant activity by DPPH assay

Antioxidant activity of different extracts of wild pomegranate was determined by DPPH assay and EC50 value was calculated. Highest antioxidant activity was recorded in red fruits (EC50 =70.33µg/ml) followed by green fruit (EC50 =111.51µg/ml), flower (EC50 = 123.51µg/ml) while it was lowest in leaves (EC50 = 120.78µg/ml). The EC50 value for BHT taken as reference was 23.5 ± 0.551µg/ml (Fig 5). Singh et al. reported the highest antioxidant activity in methanolic extracts of peels and seeds of cultivated pomegranate using various in vitro models while Shiban et al. observed the highest DPPH scavenging activity in methanol extracts of fruit peels.

FRAP assay

The antioxidant potential of four extracts of wild pomegranate was determined from their ability to reduce 2,4,6 – tripyridyl-s- triazine (TPTZ) –Fe (III) complex to ferrous form TPTZ –Fe (II) which has an intense blue colour which can be observed by measuring the change in absorption at 593nm. More the blue colour, more is the reducing power. Maximum antioxidant activity was observed in red fruits (310.99 ± 0.98 µmol Fe2+/g of extract) while it was 253.99 ± 0.67 µmol Fe2+/g of extract in green fruit. However, the antioxidant potential in flower was 95.99 ± 0.31 µmol Fe2+/g of extract while in leaves, it was 69.99 ± 0.45 µmol Fe2+/ g dry matter (Figure 3). Wang & Lin also observed a linear correlation between fruit ripeness and increase in antioxidant potential of fruit. The increase in antioxidant potential is attributed to...
increase in content of flavonoids and anthocyanins during ripening process. Hajimahmoodi et al. observed the higher FRAP values in peel extracts of cultivated pomegranate which contributed to its higher antioxidant activity.

**Conclusions and future prospects**

The present study proves the use of wild pomegranate plant parts as potential sources of natural antioxidants with efficient antioxidant potential. The high antioxidant activity of the methanolic extracts is due to the presence of phenolics and tannins. So, the plant parts can be used as potent source of secondary metabolites in pharmaceutical industries.

**ACKNOWLEDGEMENTS**

The authors are thankful to INSPIRE fellowship and DST, New Delhi for providing the financial support as major research project on wild pomegranate. The authors are also thankful to School of Biotechnology, University of Jammu for providing the basic facilities to carry out this research work.

**REFERENCES**