

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

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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 70.33 $\mu$ g/ml as compared to other tissues. The ferric reducing potential was significantly higher in red fruit (310.99  $\pm$  0.98  $\mu$ mol Fe<sup>2+</sup>/ 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.

Plants produce a wide range of bioactive molecules referred as secondary metabolites which are used as a source of medicines for treatment of various diseases. Secondary metabolites apart from being safe for human consumption are also environment friendly. Most of the medicinal plants species present in India are used as potent therapeutic agents. Epidemiological studies reveal that consumption of fruits and vegetable rich in phytochemicals, reduces the risk of cancer and chronic degenerative disorders<sup>1,2</sup>.

Wild pomegranate is an ancient plant which may have been originated from Iran. In India, wild pomegranate grows only in the three

regions of north western Himalayas (Jammu and Kashmir, Himachal Pradesh and Uttarakhand)<sup>3</sup>. The commercial use of wild pomegranate is in the form of anardana which is used as acidulent in culinary preparation like curries and chutneys. Most of the parts of the plant are rich in phytochemicals that further contributes to health promoting benefits<sup>4</sup>. Rind and arils of cultivated and wild pomegranate are rich in ellagic acids, flavonoids, anthocyanins and hydrolyzable tannins<sup>5,6</sup>.

In context to above apprehensions, the present study was undertaken to study different parts of wild pomegranate plant for its phytochemical constituents and antioxidant properties.

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## 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% HgCl<sub>2</sub> 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<sup>7,8</sup>.

#### Test for carbohydrates

One ml of the each extracts was added to 2 ml of molish reagent ( $\pm$ -naphthol in 5% alcohol), followed by the addition of 1 ml concentrated H<sub>2</sub>SO<sub>4</sub>. Formation of red or dull violet 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 tube followed by addition of few drops of 1% copper sulphate. 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<sup>9</sup>. 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<sup>10</sup>. 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-Ciocalteu (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-ciocalteu assay<sup>11</sup>. 1N folin -ciocalteu reagent was freshly prepared and added to 100  $\mu$ l 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.

Gallic acid was used as standard. Concentration of phenolics of each sample was determined by the following equation:

$$\text{Absorbance} = 0.0364 \text{ gallic acid } (\mu\text{g}) + 0.009$$

#### Determination of total tannins

Total tannin content was estimated using method described by Rebaya *et al.*<sup>12</sup>. 100  $\mu$ l methanolic extract of each sample was mixed with 3 ml vanillin solution followed by the addition of 1.5 ml concentrated HCl. The mixture was incubated for 5 min and absorbance was taken at 500nm. Tannic acid was used as standard.

#### Determination of antioxidant activity

The antioxidant activity of all four samples was studied using DPPH assay and FRAP assay.

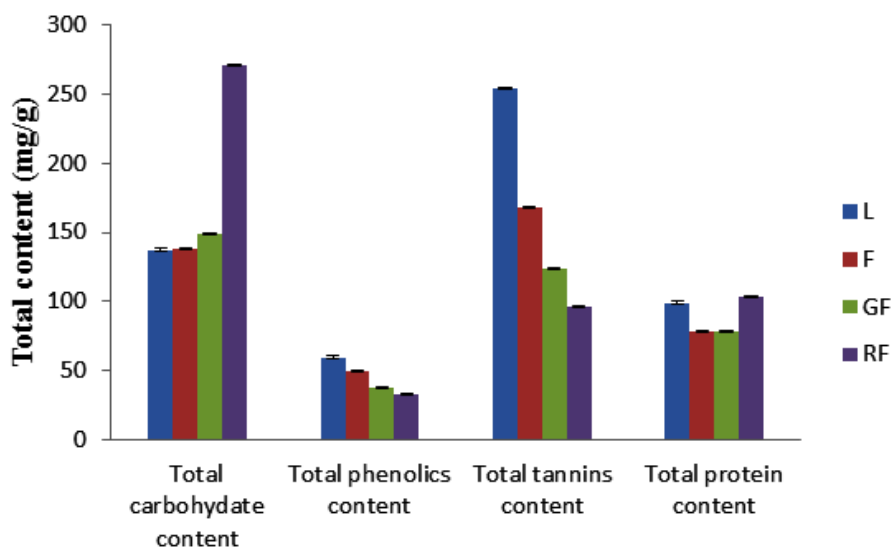
#### 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH radical scavenging activity was determined by protocol as described by Ebrahimzadeh *et al.*<sup>13</sup>. The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical is very stable which reacts with compounds having weak A-H bond. Reduction of DPPH by the antioxidants present in extracts results in decolourization of DPPH methanol solution. DPPH reagents were prepared by mixing 18 mg of DPPH in 100 ml of methanol. The 3  $\mu$ l extracts were mixed with 100  $\mu$ l of DPPH followed by addition of 2 ml of acetate buffer. The tubes were incubated at room temperature in the dark for 30 min and absorbance was recorded at 517nm. BHT was used as a reference. The results were expressed as percentage reduction of the

**Table 1.** Qualitative analysis of phytochemicals in wild pomegranate extracts

S.No.	Phytochemical tests	Green fruit	Red fruit	Flower	Leaf
1.	Terpenoids	++	++	++	+
2.	Saponins	++	+	+	+
3.	Steroids	++	++	++	+
4.	Flavonoids	++	+++	++	++
5.	Coumerins	++	++	++	+
6.	Tannins	++	+	++	+++
7.	Proteins	++	+++	++	++
8.	Carbohydrates	+	+++	+	+
9.	Phenolics	++	+	++	+++

(+++)= 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.

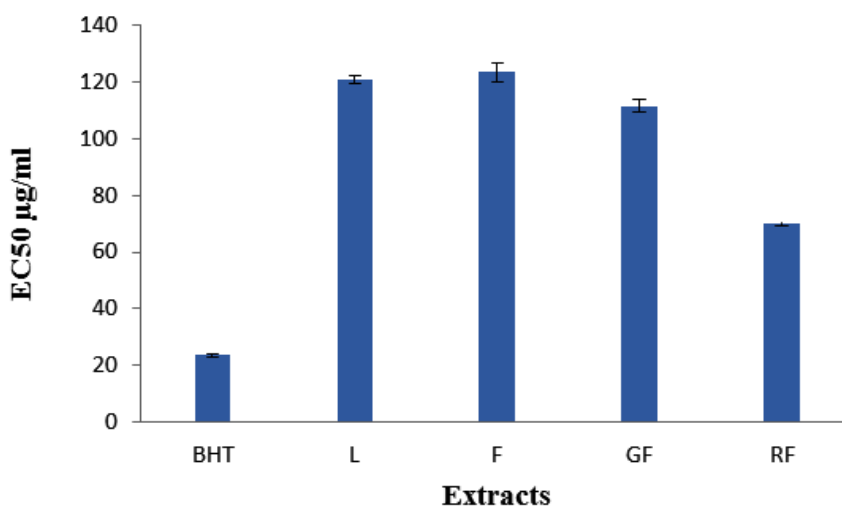
#### Ferric Reducing Anti-oxidant Power (FRAP assay)

Antioxidant activity of four methanolic extracts was assessed by FRAP assay<sup>14</sup>. It is based on the principle of reducing ferric to ferrous ions at low pH that results in the formation of a coloured ferrous–tripirydyltriazine complex. FRAP reagents includes 300 mmol/l acetate buffer (pH 3.6),

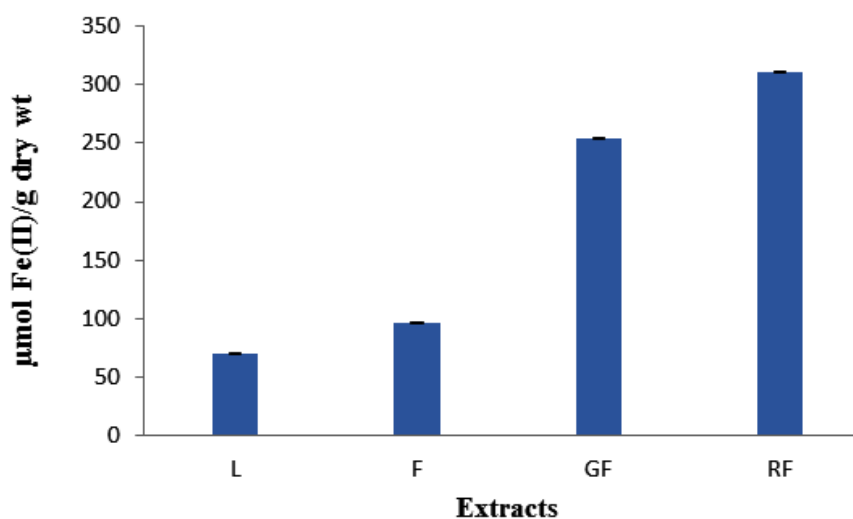
10mmol/l TPTZ in 40 mmol/l HCl and 20mmol/l FeCl<sub>3</sub>. 3 ml of the FRAP reagent was added to 100 µl of each extract and incubated at 37p C for 5 min. Absorbance was taken at 593nm using FeSO<sub>4</sub> as the standard solution.

#### 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<sup>15</sup>. Many plants rich in flavonoids, alkaloid and terpenoids have anti-cancerous properties<sup>16</sup>.

### 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<sup>17</sup>. These results are in agreement with Zarie *et al.*<sup>18</sup>, 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-Ciocalteu 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<sup>19</sup>.

#### 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<sup>20</sup>.

#### 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<sup>21</sup>.

#### 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-picrylhydrazyl) 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 EC<sub>50</sub> value was calculated. Highest antioxidant activity was recorded in red fruits (EC<sub>50</sub> = 70.33 µg/ml) followed by green fruit (EC<sub>50</sub> = 111.51 µg/ml), flower (EC<sub>50</sub> = 123.51 µg/ml) while it was lowest in leaves (EC<sub>50</sub> = 120.78 µg/ml). The EC<sub>50</sub> value for BHT taken as reference was 23.5 ± 0.551 µg/ml (Fig 5). Singh *et al.*<sup>22</sup>, reported the highest antioxidant activity in methanolic extracts of peels and seeds of cultivated pomegranate using various *in vitro* models while Shibani *et al.*<sup>23</sup> 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 Fe<sup>2+</sup>/g of extract) while it was 253.99 ± 0.67 µmol Fe<sup>2+</sup>/g of extract in green fruit. However, the antioxidant potential in flower was 95.99 ± 0.31 µmol Fe<sup>2+</sup>/g of extract while in leaves, it was 69.99 ± 0.45 µmol Fe<sup>2+</sup> / g dry matter (Figure 3). Wang & Lin<sup>24</sup> 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.*<sup>25</sup> 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.

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