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Variation in secondary metabolite content between peel of green and red fruits of wild pomegranate of Dhera ki Gali from Jammu division in India

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Abstract
Cultivated pomegranate is known for its secondary metabolites and antioxidants so does its wild variety i.e. wild pomegranate. In this study, secondary metabolites and antioxidants were determined from fruits (both green and red) of ten trees of wild pomegranate from Dhera ki Gali of Jammu province. It was found that red fruits have shown more metabolite content as compared to green fruits and peel part of fruits showed more activity as compared to pulp part. On comparing ten trees, it was also found that fruits of trees of upper reaches have shown more activity as compared to lower reaches indicating the impact of climatic factors on these fruits.

Keywords: Wild pomegranate, altitude, secondary metabolites, antioxidants, green fruits, red fruits, peel.

Introduction
Plants being sessile, have evolved several mechanisms for accommodating the changes that arise due to variable growth conditions and to enable them to survive under the sway of many environmental factors without affecting their own cellular and physiological developmental processes (Arnold *et al*. 2019) [1]. In response to varying environmental conditions that includes local geo-climatic changes, light, humidity, external temperature and developmental processes, plants synthesize secondary metabolites (Zykin *et al*. 2018) [22]. Further, these secondary metabolites produced by the plants are not of much importance in their primary life as the production is at low concentration which is appropriate for growth and physiology of the plants (Isah 2019) [13]. These metabolites help the plants to adapt and cope up with stressful constraints during changing environments. Large number of metabolites produced by the plants are accumulated in plant cells and their production can be induced *in vitro* also, by exposing the cells to various biotic and abiotic elicitors which has exploited for making drugs or herbal medicine to cure various ailments (Asgher *et al*. 2020, Wurtzel and Kutchan, 2016) [2, 21]. Several external factors and internal factors such as Season, climate, temperature, microbial attack, radiation, nutritional status, grazing, competition and various genetic factors have a strong influence on the metabolite content in the different parts of the fruits. The current work was designed to the secondary metabolite and antioxidants in the wild pomegranate grown in higher altitudes of Jammu division.

Materials and Methods
Collection of plant materials
The plant species under study is *Punica granatum* L. Fresh leaves, green and red fruits of wild pomegranate were collected from upper and lower reaches of Dhera Ki Gali. Green fruits and leaves were collected during June and July whereas the red fruits were collected during October and November in the year 2013. 7-8 fruits from 10 trees were collected. All the collected fruits (green and red) were washed and peeled. Their peel was separated and kept for drying in an oven at an average temperature of 35-36 °C. Samples were dried in the oven for 3-5 days till they completely dried. After drying the peel of both green and red fruits, samples were further crushed and grinded with the help of mortar and pestle and the final powdered form was obtained using an electric grinder. The number of replicates was 10 (n=10). The secondary metabolites and antioxidant metabolism was studied.

Quantitative Phytochemical Analysis
Quantitative estimation was done from the extracts prepared from peel of green and red fruits.

Total Phenolic content

Total phenolic content was determined by the procedure given by Singleton and Rossi (1965) [19] using Folin-Ciocalteu reagent. In this procedure, 50 µl of extract solution was taken and 950 µl of water was added to it. Then 0.5 ml of 1N FC reagent was mixed and kept at room temperature for 5 min. To it 2ml of 7.5% sodium carbonate was added to this reaction mixture. Then the tubes were incubated in the dark for 1 hour and after that O.D was taken at 750 nm. Gallic acid was used as standard and the results were expressed as µg gallic acid equivalent (µg GAE/g extract).

Total flavonoid content

Total flavonoid content was measured spectrophotometrically using aluminum chloride colourimetric method as per protocol given by Chandra *et al.* (2014) [7]. This method is based on the formation of flavonoid–aluminium complex, having maximum absorbance at 510 nm. Plant methanolic extract was diluted 2.5 ml of distilled water and then 150 µl of NaNO2 was added to it. After incubation of 5 minutes at room temperature, 300µl of 10% AlCl3H2O was added to the reaction mixture. After 6 min, 300 µl of 1 M NaOH and 550 µl of distilled water were added to the reaction mixture and the absorbance was observed at 510 nm using UV-VIS spectrophotometer. Quercetin was used as standard and the total flavonoid content was determined using a standard curve and was expressed as milligram Quercetin equivalents per gram of extract (mg QE/g extract).

Total tannin content

Total tannins were determined by the method of Elfalleh *et al.* (2012) [9]. To 1 ml of the diluted extracts, 5 ml of 2.5% KIO_3 was added into a tube and vortexed for 10s. The tubes were incubated at room temperature for 2 minutes. Absorbance of the red-colored mixture was determined at 550nm. Tannic acid was used as reference. The final results were expressed as mg tannic acid equivalent per g of extract (mg TAE/g extract).

Anthocyanin estimation

The spectrophotometric method for determining the anthocyanin content using different pH is called as Anthocyanin pH differential method by Lee *et al.* (2005) [15]. Anthocyanin is pH dependent, at pH 1.0 anthocyanin are colored and transmit various wavelengths and at pH 4.5 all of them are colorless. Cyanidin-3-glucoside (26,900) was used to calculate the anthocyanin content. The results were calculated as mg/100ml of cyanidin - 3- glucoside by using molar absorptivity of 26,900.

Solutions for monomeric anthocyanin pH differential method

A. **pH 1.0 buffer (Potassium chloride 0.025M):** Dissolved 1.86 gm of KCL in 980 ml of distilled water. Took the pH and adjusted the pH to 1.0 by adding 6.3ml of HCL in it. Transferred the solution to volumetric flask and diluted it with distilled water.
B. **pH 4.5 buffer (Sodium Acetate 0.4M):** 54.53 gm of sodium acetate was dissolved in 960 ml of distilled water. pH of 4.5 was adjusted by adding 20 ml of HCL in the solution. Transferred the solution to volumetric flask and diluted it with distilled water.

Sample preparation

Weighed 100 mg of powdered sample and put it into 100ml of volumetric flask. Added 80 ml of distilled water to the flask. Sonicated the solution for 15 minutes and raised the volume to 100 ml. Allowed to cool and mix. Took 1ml of the aliquot of the extract solution and put it into 10 ml test tube. Diluted it by adding 9 ml of buffer (pH 1.0) and mixed gently. Remove a second 1ml of aliquot of the solution and placed in another 10ml test tube and diluted it by adding 9 ml of buffer (pH 4.5) and mixed well. Solution was stable for 4 hours at room temperature.

Protocol

Took 1 ml of extract in 10 ml of volumetric flask for the preparation of two dilutions of samples. One dilution was prepared with potassium chloride buffer having pH 1.0 and other dilution was prepared in sodium acetate buffer having pH 4.5. Allowed the solutions to equilibrate for 15 minutes. Then the absorbance of each solution was measured at 510 nm and 700 nm against blank set with distilled water. All measurements were made between 20 minutes and 1 hour after the preparation of samples as longer standing time of samples leads to increase of reading observed. The samples to be measured should be clear and should contain no sediments or haze, though little bit of colloidal material could remain suspended in the samples that cause cloudy appearance due to scattering of light. This scattering of light should be corrected by measuring the absorbance at 700nm where no scattering or absorption of light can occur. Absorbance of the samples was calculated as

Absorbance (A) = (A510- A700) pH 1.0 - (A510-A700) pH4.5

Anthocyanin pigment concentration is expressed as cyanidin –
3- Glucoside equivalents. W/W = A× MW ×DF×V/ Ɛ×L×Wt×100

A – Absorbance

Ɛ – Cyanidin 3 glucoside absorbance (26900) MW – Anthocyanin Molecular weight (449.2) DF- dilution factor

V- Final volume (100 ml).

Wt. – Sample Weight (100 mg) L – cell path length (1 cm).

***In vitro* Antioxidant studies**

The crude extracts from peel and pulp of green and red fruits from five locations were analyzed for antioxidant activity which are described below:

Ferric ion reducing antioxidant power (FRAP assay)

Ferric reducing antioxidant power assay is the method for assessing total antioxidant power, given by Benzie and Strain (1996) [3]. The antioxidants act as Reductant and reduce the free radicals or free electrons of the reaction sample in redox-a linked colourimetric reaction in which Fe^{3+} get reduced to Fe^{2+}. This reduction of the target compounds leads to the production of a dark blue or purple-coloured ferrous complex from a colourless ferric complex. O.D was taken at 593nm.

Protocol

Extracts of three different concentrations (10 µl, 20 µl, 30 µl) was taken from the aliquots and added in three different test tubes. After that 990 µl, 980 µl, and 970 µl of methanol were added to these tubes respectively. Reagents of FRAP were prepared by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of ferric chloride solution. 3ml of FRAP reagent was added to each of the test tubes. The test tubes were incubated at 37 ºC for 5 minutes. After that O.D was taken at 593nm. The blank was set up with methanol in equal volume instead of plant material. Trolox was taken as standard and the standard curve was obtained by using the concentrations ranging from 100 to 500 mg\L prepared in distilled water. The results obtained after going through this protocol were shown as ascorbic acid equivalents per ml.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay is used to determine antioxidant activities by the mechanism in which antioxidants act and inhibit lipid oxidation, so scavenging of DPPH free radical and therefore determinate free radical scavenging capacity.

In this method, DPPH solution is mixed with a substance that can donate a hydrogen atom and reduce it to 2-2-diphenyl-1-hydrazine, changing the violet colour into a straw yellow-coloured solution. Antioxidants react with DPPH, reducing the number of DPPH molecules equal to their number of available hydroxyl groups.

The DPPH radical-scavenging activity was determined by the method of Brand-Williams *et al.* (1995) [6]. Trolox was used as positive control and methanol as negative control and extract without DPPH was used as blank. Results were expressed as a percentage reduction of the initial DPPH absorption in relation to the control. The concentration of extract leading to 50% reduction of DPPH (IC50) was also determined.

Solutions for DPPH assay

A. DPPH – 18 mg of DPPH was dissolved in 100 ml of methanol.
B. Acetate buffer – 0.410 g of sodium acetate was dissolved in 50ml distilled water and 115 µl of glacial acetic acid was dissolved in 50ml distilled water respectively. Then took 41.2 ml 0f sodium acetate and 8.8 ml of glacial acetic acid and added 50 ml of distilled water to raise the volume. Then filtered it and stored it in the refrigerator.

Result and discussion

Variation in phenolic content in peel

On comparing the variation in phenolic content between green and red fruits peel of ten trees, the range was from 86.3±3.12-126.7±6.62 mg GAE/gm for green peel and it was from 140.5±9.17-249.7±7.40 mg GAE/gm for red peel (Table 1; Fig 1). Phenolic content was observed to be more in red fruit peel as compared to green peel.

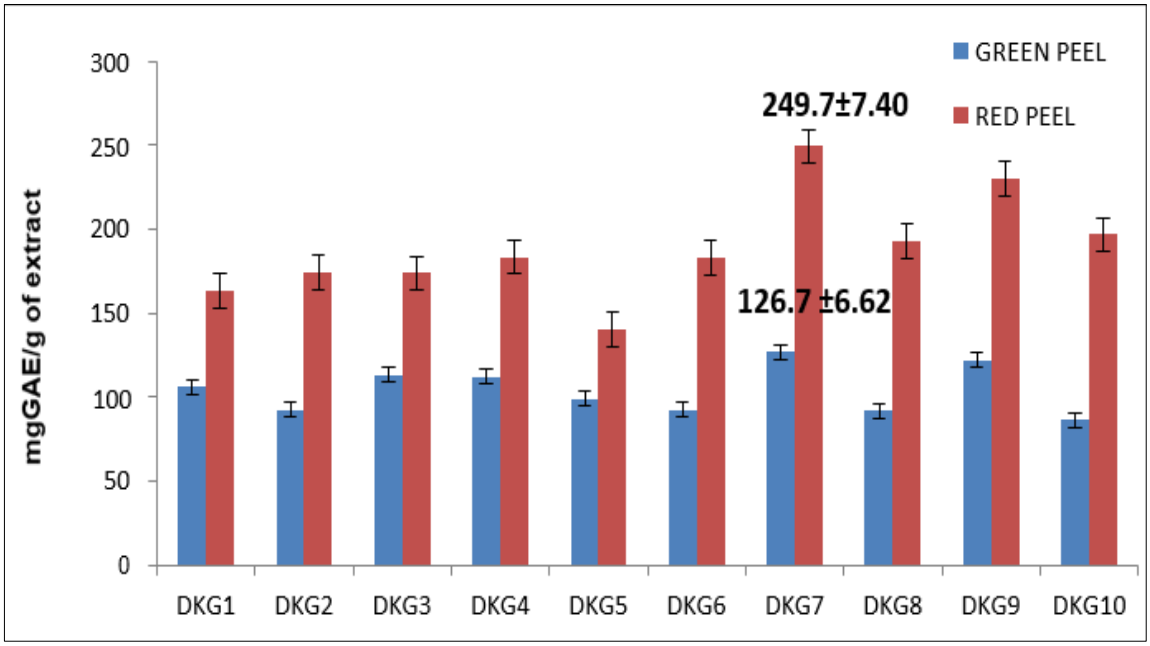

Fig 1: Variation in phenolic content in peel of green and red fruits

Variation in flavonoid content in peel

Similarly, flavonoid content variation was observed in green and red fruit peels of ten plants. The range was between 205.2±5.12 -305.3 ±9.17 mgQE/gm and 248±8.12 -386.1±12.18 mgQE/gm extract for green and red fruit peel respectively (Table 1; Fig. 2). Flavonoid content was more in red fruit peel as compared to green peel.

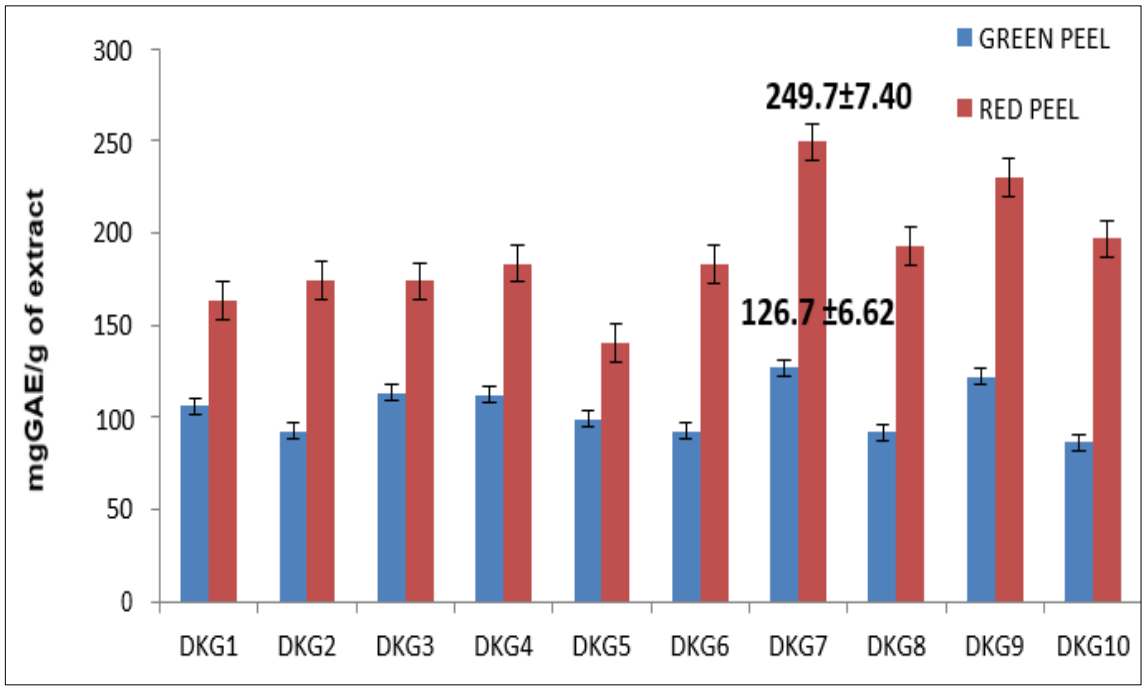

Fig 2: Variation in flavonoid content in the peel of green and red fruits

Variation in tannin content in peel

Variation in tannin content in green fruit peel ranged from 113.6±8.30, -184.1±7.30mgTAE/gm extract whereas the content in red peel ranged from 80.9±8.3, -132.4±6.30 mgTAE/gm (Table 1, Fig. 3).

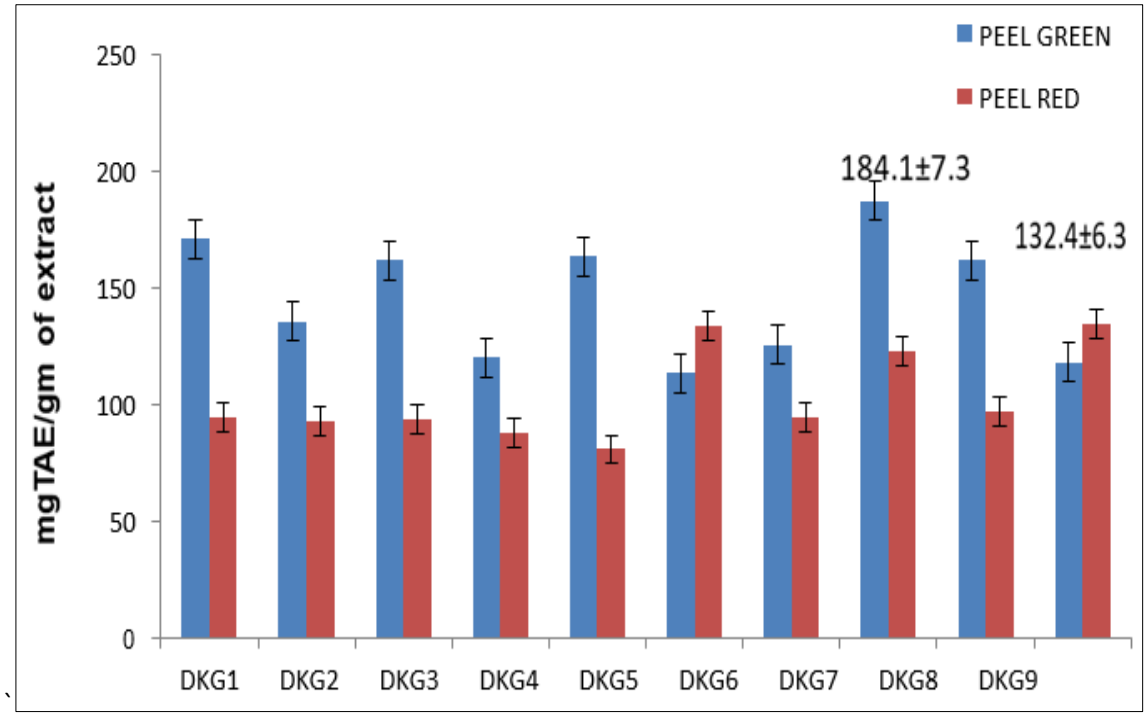

Fig 3: Variation in tannin content in the peel of green and red fruits

Variation in anthocyanin content in peel

The variation in anthocyanin content in the green fruit peel of ten plants ranged from 8.33±0.1 -17.1±1.10mg CGE/gm whereas in red fruit peel, the range was from 18.86±2.01-36.4 ±2.10mg CGE/gm. (Table 1, Fig. 4).

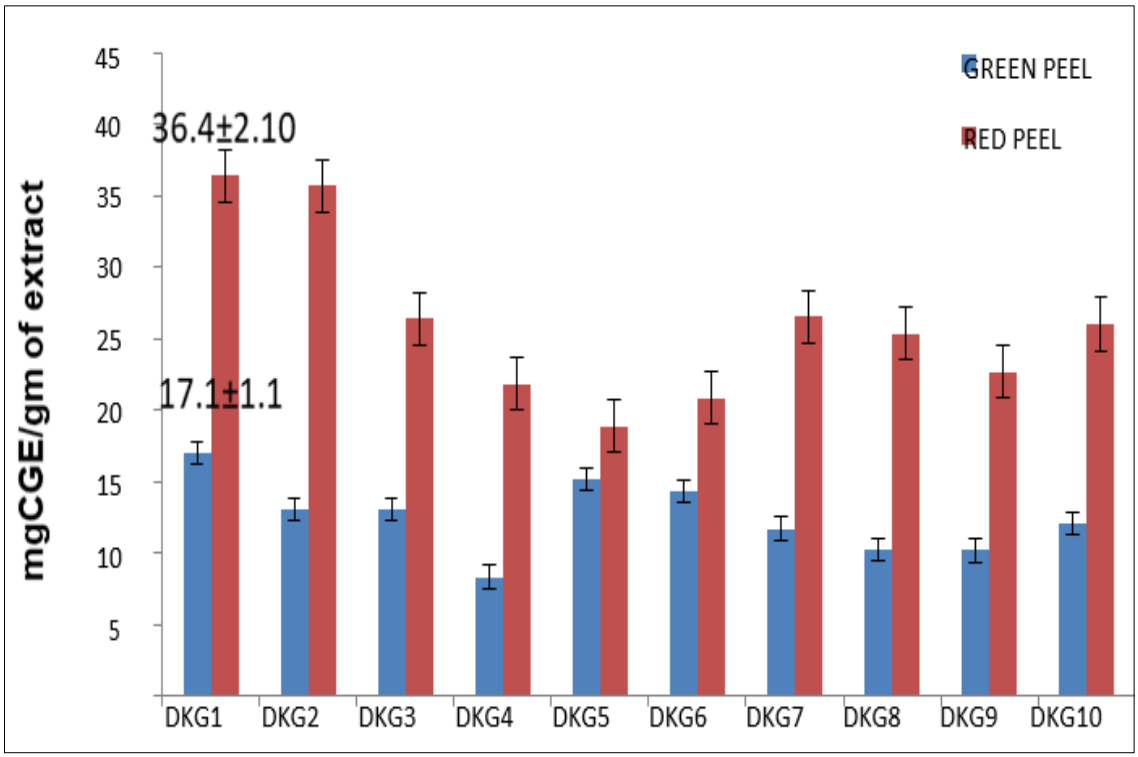

Fig 4: Variation in anthocyanin content in peel of green and red fruits

Variation in antioxidant activity in peel

Using, FRAP assay, the antioxidant activity (ferric reducing ability) in the green peel of fruits ranged from 3210.6±26.2 - 4282.6±36.18 µm Fe(II)/gm and in red peel the range was from 3808.6±32.61 - 6708.6±43.12 µm Fe(II)/gm. However, a significant difference in FRAP activity for green and red fruit peel among different altitudes was observed (Tables 1, Fig 5).

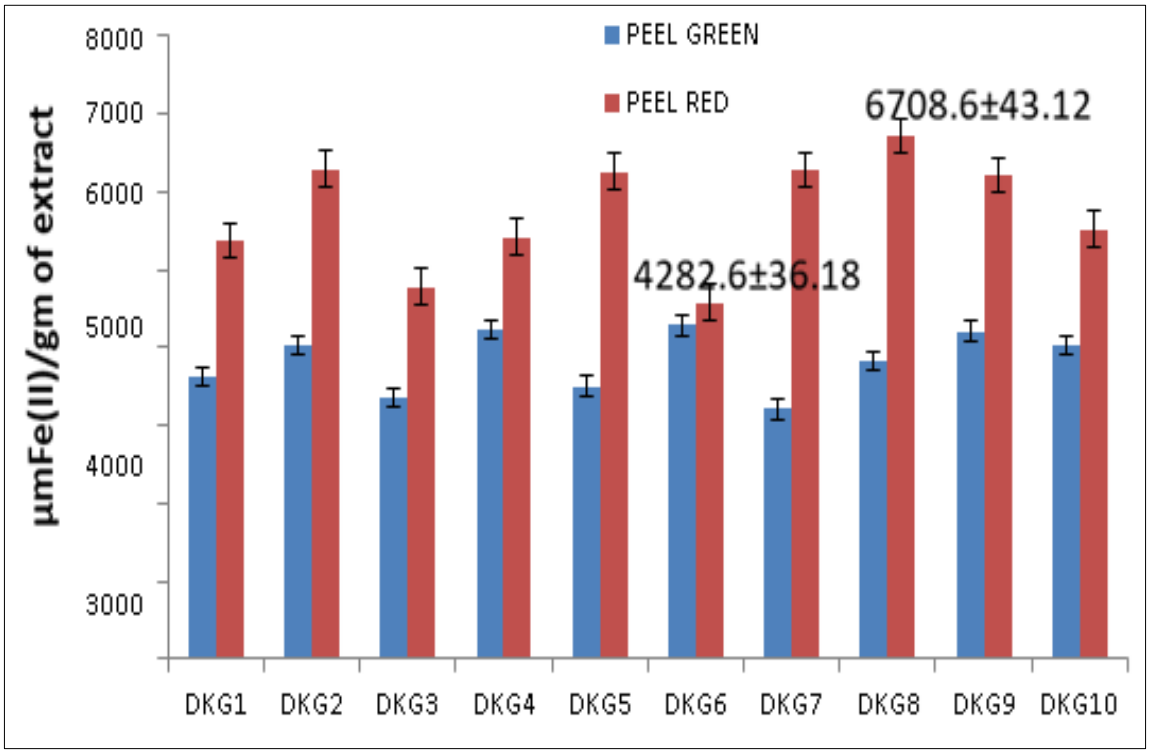

Fig 5: Variation in antioxidant activity (FRAP) in the peel of green and red fruits

Green and red fruits had shown variation in DPPH free radical scavenging in the range 17.1±0.2 - 21.1±1.12µg/ml for green fruit peel and for red peel it ranged from 14.8±0.40-19.45±1.06µg/ml (Tables 1, Fig 6).

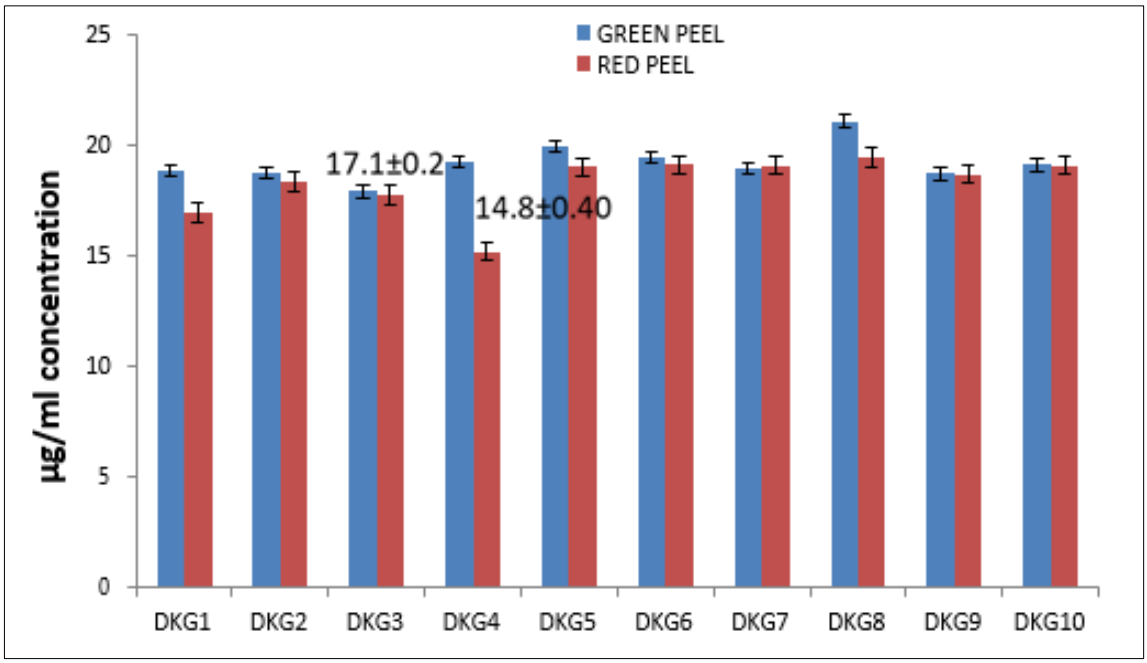

Fig 6: Variation in free radical scavenging activity (DPPH) in peel of green and red fruits

Discussion

It was observed that fruits of wild pomegranate collected from plants of upper reaches of Dhera Ki Gali (DKG5, DKG6, DKG7, DKG8) generally showed more antioxidant (DPPH and FRAP) and secondary metabolite content as compared to plants of lower reaches and red fruits showed more metabolite content as compared to green fruits except for tannin content which was more in green fruits as compared to red fruits.

However, Solarte *et al*. (2014) [20] reported that the altitude influenced the ripened fruit weight in different genotypes of guava. Similarly, Gonzalez *et al*. (2011) [12] also reported that the temperature of a region influences the secondary metabolites and colour content of fruits at higher altitudes. Gautier *et al*. (2008) [11] also reported that variations in temperature influenced the flavonoid content in plants. Similarly, the various environmental factors like day and night temperature, solar radiations, and atmospheric pressure influenced the fruit ripening and also improved the skin colour in peaches (Karagiannis *et al*. 2016) [14].

Similar types of results were also found by Elloumi *et al*. (2013) [10] in which apple fruits collected from higher altitudes i.e. 2780 m showed the highest antioxidant and polyphenolic content as compared to lower altitudes locations.

The variation in total phenolics content among the plants within the location and between the locations could be attributed to geographic sources of samples and their altitudinal variations.

These variations in metabolites reflect the differences in climatic conditions such as high temperature, water stress conditions, variations in ultraviolet rays and nutritional differences with respect to plants growing at different altitudinal locations (Berli *et al*. 2011) [4]. Besides, Moretti *et al*. (2010) [17] suggested that plants and their products i.e vegetables and fruits grown under more stress conditions like higher temperature and more CO2 concentration produced higher bioactive compounds like polyphenolics and ascorbic acid.

Marsic *et al*. (2011) [16] and Drogoudi and Pantelidis (2011) [8] also reported that plants of cooler regions at higher altitudes showed rich phenolic and antioxidant content due to more generation of anthocyanin content. The fruit position i.e. fruits which are more exposed to sun radiations showed highest pigment produced by anthocyanin. Similar results are also presented by Rawat *et al*. (2016) [18] and Bahukhandi *et al*. (2018) [5] which showed that secondary metabolites like polyphenolics, anthocyanins, flavonoids and antioxidants are higher in plants growing at higher altitudes.

Table 1: Metabolite content in green and red fruit peel collected from Dhera ki Gali

Plant No	Green fruits (Peel)						Red fruits (Peel)					
	Phenolics (mgGAE/gm extract)	Flavonoids (mgQE/ gm extract)	Anthocyanins (mgCGE/gm extract)	Tannins (mgTAE/gm extract)	Antioxidant activity		Phenolics (mgGAE/gm extract)	Flavonoids (mgQE/ gm extract)	Anthocyanins (mgCGE/gm extract)	Tannins (mgTAE/gm extract)	Antioxidant activity	
					FRAP (µmFe (II)/gm extract)	DPPH (µg/ml)					FRAP (µmFe (II)/gm extract)	DPPH (µg/ml)
DKG 1	106.3±4.62	305.3±9.17	17.1±1.10	170.9±6.32	3615.6±33.02	18.84± 0.72	163.5±7.40	344.9±12.32	36.4±2.10	94.54±3.3	5364.6±42.23	16.95±2.26
DKG 2	92.4±5.37	235.1±8.27	13.06±0.66	135.45±5.43	4030.6±34.22	18.74± 0.64	174.3±8.15	302.3±11.28	35.73±2.90	92.72±7.13	6290.6±40.07	18.35±1.3
DKG 3	113.3±6.69	208.2±9.07	13.07±0.73	161.81±7.21	3354.6±31.53	17.1±0.20	174.0±8.42	342.8±12.32	26.38±3.10	93.63±9.31	4780.6±30.21	17.76±3.06
DKG 4	112.4±5.60	258.7±8.13	8.33±0.10	120.1±4.15	4226.6±31.71	19.23± 0.93	183.7±7.43	342.1±13.18	21.87±1.51	88.1±8.30	5416.6±40.01	14.8±0.70
DKG 5	99.1±5.60	212.1±12.3	15.1±0.90	163.6±7.53	3505.6±33.79	19.97± 0.89	140.5±9.17	380.0±10.18	18.86±2.01	80.9±6.30	6256.6±35.21	19.03±1.56
DKG 6	92.4±8.16	212.7±8.21	14.3±0.81	113.6±8.30	4282.6±36.18	19.46± 1.02	183.2±9.12	386.1±12.18	20.87±1.10	130.6±9.13	4576.6±40.2	19.13±1.46
DKG 7	126.7±6.6	226.4±7.21	11.7±0.62	125.4±6.71	3210.6±26.20	18.94± 0.99	249.7±7.40	362.2±9.32	26.55±3.13	94.54±7.41	6286.6±40.80	19.09±1.96
DKG 8	92.3±6.60	236.1±7.90	10.2±0.43	184.1±7.30	3822.6±32.17	21.1± 1.12	193.2±7.49	336.9±13.30	25.38±2.50	122.72±8.90	6708.6±43.12	19.45±1.06
DKG 9	122.1±8.43	205.2±5.12	10.1±0.41	161.8±5.85	4210.6±37.01	18.72± 1.06	230.2±8.43	282.2±13.21	22.71±2.32	96.89±79.13	6212.6±34.21	18.67±2.86
DKG 10	86.3±3.12	213.7±6.20	12.09±0.67	118.1±9.01	4016.6±35.40	19.12± 1.11	197±7.31	248.1±8.12	26.05±2.53	132.4±6.30	5518.6±43.20	19.07±1.9

Values represent Mean ± Standard error of three replicates

Conclusion

The purpose of the current study was to determine the variation in metabolites in red and green fruits of ten different plants of wild pomegranate from Dhera ki Gali of Jammu region. The study showed a high variation of the measured secondary metabolites among 10 populations. Among them the phenolic, flavonoid and anthocyanin content of red fruits was significantly more as compared to green fruits. Also, the plants at higher reaches showed more metabolite contents. Therefore, it is recommended that plants of Dhera ki Gali shall be selected for future programmes for pharmaceutical purposes.

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