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Assessment of phytochemicals and phenylalanine ammonia Lyase activity of *Punica granatum* L.

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Abstract
Pomegranate fruit gained lot of attention for its commercial propagation due to presence of nutritionally and beneficial bioactive compounds in relation to various health issues in worldwide. The important horticulture and quality traits to hike the market value of pomegranate includes fruit size, colour, aril size, Juiciness and soft seed. Hence, the present investigation was aimed to determine the bioactive compounds and Phenylalanine ammonia lyase activity in different cultivars of pomegranate with varied seed type. During study total PAL activity was found to be ranged from 57.94 ± 0.12 to 158.56 ± 0.50 U/ml in crude aril extract of different pomegranate varieties. The total phenol content in the methanolic aril extract was ranged from 2.96±0.72 to 14.22±0.05GAE mg/g fresh aril weight. The maximum value for total phenolic content was reported in soft seeded variety Phulle Super Bhagwa. Significantly highest total flavonoid content (12.93±0.06QE mg/g fresh aril weight) and carbohydrate content (38.95±0.35 DE mg/g fresh aril weight) was reported in methanolic aril extract of Kandhari Kabuli. The results obtained during the present studies showed wide variability in the total phenol, flavonoid and carbohydrate content and phenylalanine ammonia lyase activity among different cultivars. Total phenolic content and activity of PAL enzyme was positively maximum in softseed variety (Phulle Super Bharwa) and Flavonoid and Carbohydrate content was reported to be maximum in Kandhari Kabuli.

Keywords: Phytochemical, phenylalanine ammonia lyase, pomegranate aril, secondary metabolites

1. Introduction
Pomegranate is a known as king of subtropical fruits with high nutraceutical properties. Pomegranate is a multipurpose, fast-growing species of economical and ecological importance. The edible parts of pomegranate fruits are consumed fresh or used for the preparation of fresh juice, canned beverages, jelly, jam, paste and also for flavoring and coloring beverage products (Fadavi *et al*., 2005; Mousavinejad *et al*., 2009) [14, 23]. About 100 g arils provides 72 kcal of energy, 1.0 g protein, 16.6 g carbohydrate, 1 mg sodium, 379 mg potassium, 13 mg calcium, 12 mg magnesium, 0.7 mg iron, 0.17 mg copper, 0.3 mg niacin and 7 mg vitamin C (Grove and Grove, 2008) [16]. The fruit is a good source of sugar, vitamin C and iron (Prasad and Mali, 2000) [28].
The pomegranate fruit has also got various medicinal properties due to the presence of various phytochemicals. Phytochemicals are wide range of compounds naturally present in plants and possess therapeutics properties as imparts a main role in shielding and curing various chronic diseases such as cancer, hypertension, heart disease, diabetes and other medical conditions. On the basis of chemical structure and features these phytochemicals have been categories into six classes which include phenolics, carbohydrate, terpenoids, lipids, and alkaloids, and other nitrogen-containing compounds (Campos and Oomah, 2013) [6]. Each class is sub divided based on biosynthetic origin which includes metabolites like tannins, alkaloids and flavonoids; known to have medicinal properties in plants (Bhandary *et al*., 2012) [4]. Plants are major source of secondary metabolites which are formed as products of primary metabolism and produced for defense against predators (Unuofin *et al*., 2017) [34].
Since ancient times, the pomegranate has been regarded as a "healing food" with numerous beneficial effects in several diseases (Vidal *et al*., 2003) [35]. This fruit is laxative, diuretic and used for curing vomiting, sore throat, brain diseases, spleen complaints, chest troubles, scabies, bronchitis, liver and kidney disorders (Kirtikar and Basu, 1985; Mena *et al.*, 2012) [18, 22]. Its fruit extracts are being used to treat several parasitic and microbial infections, diarrhea, ulcers, apthe, hemorrhage and respiratory complications (Naqvi *et al*., 1991 and Caceres *et al*., 1987) [25, 5]. Extracts of different parts of the fruit seem to have medicinal benefits (Lansky and Newman, 2007) [19].

Pomegranate juice contains polyphenols (primarily ellagic and punicalagin) that may lower down the risk of heart diseases, neurological diseases and slow down the cancer progress (Adams *et al*., 2006) [1]. Seeds of this fruit also have effective antimicrobial activity against various microorganisms (Sagdic *et al*., 2010) [30]. The peel extracts of pomegranate have important anti-bacterial activities (Devi *et al*., 2011) [11] against *Staphylococcus aureus, Listeria monocytogens, Escherichia coli, Yersinia enterocolitica* and *Salmonella enteritidis* (AI, 2009) [2].

Pomegranate has a great antioxidant potential due to high levels of phenolic compounds, flavonoids, anthocyanins, tannins, ascorbic acids and gallic acid present in the fruit (Salgado *et al*., 2012; Kaur *et al*., 2018; Redha *et al*., 2018; Derakshan *et al*., 2018) [31, 17, 29, 10].The phytochemistry of pomegranate has also been widely studied by number of researchers and pomegranate is found to be a rich source of polyphenolic compounds (Dandekar *et al*., 2008) [9]. Both flavonoids and tannins are more abundant in the peels (Ozcal and Dinc, 1993) [27]. Peels of pomegranate contain a wide variety of phytochemical compounds like gallotannins, ellagic acid, gallic acid, punicalins, punicalagins.

Phenylalanine ammonia lyase is one of the key enzyme during phenyl propanoids biosynthesis in plants which converts L-Phenylalanine into pools of aromatic compounds which collectively known as (poly) phenolics. These aromatic compounds could be classified as flavonoids/anthocyanin, hydroxycinnamates, coumarins, phenols, lignins and phytoalexins (Vort *et al*., 2010) [36]. Many studies suggest that PAL plays an essential role in modulating the resistance of plant tissues to abiotic stresses (Wang *et al*., 2007) [37] and also provides resistance against infection by pathogens in many higher plants (Dixon *et al*., 1995; Mani *et al*., 1996 and Mur *et al*., 1996) [12, 21, 24]. This is one of the most intensively studied enzymes in plant secondary metabolism (Lewis *et al*., 1999). Hence, the present study was aimed to qualitative analysis of phytochemicals and Phenylalanine ammonia lyase activity in the arils of mature fruits of *Punica. granatum* L.

2. Materials and Methods

2.1 Screening of biological active compounds in aril of *Punica granatum* L.

2.1.1 Materials

Arils from fully mature fruits of ten different varieties of *P. granatum viz*., Kandhari Hansi (medium seeded), Kandhari Kabuli (hard seeded), Mridula (medium seeded), P-26 (medium seeded), Jodhpur Red (medium seeded), Nabha (hard seeded), G-137 (medium seeded), Chawla (hard seeded), Wonderful (medium seeded) and Phulle Super Bhagwa (soft seeded) were collected from experimental farm, Department of Fruit Science, College of Horticulture and Forestry, Neri, Hamirpur and Regional Horticulture Research and Training Station, Bajaura and stored in deep freezer for further studies. The arils from these pomegranate varieties were screened for different biological active compounds *viz*., Phenylalanine ammonia lyase, total phenolic content, total flavanoid content and carbohydrate. Chemicals used for estimation of bioactive compounds were procured from Himedia, Sigma and Merck Pvt.Ltd. The instruments used during study were refrigerated centrifuge (Remi), UV-VIS spectrophotometer (Thermo Scientific) and Deep Freezer (Thermo Scientific).

2.1.2 Extraction of crude Phenylalanine ammonia lyase from mature seeds of different varieties of *Punica granatum* L.

The extraction and assay of PAL activity was determined as per the method described by Assis *et al.*, (2001) [3]. Borate buffer (pH-8.0 was used for the extraction of crude PAL from mature seeds of Kandhari Kabuli. Seeds of different *P. granatum* varieties were grounded with the help of pestle mortar to make a fine powder. The fine seed powder was defatted with 80% acetone. 1g seed powder was mixed with 10 ml of 0.1M Sodium borate buffer to make a homogenized mixture and kept on magnetic stirrer for 1 hrs at 4°C. After 1 hrs, the homogenate was centrifuged at 13,000 rpm for 20 min at 4 °C. The clear supernatant was used for enzyme assay.

2.1.2.1 PAL (Phenylalanine Ammonia Lyase) assay:

The total PAL activity in crude aril extract was measured as per the method described by Assis *et al*., (2001) [3]. The absorbance of PAL activity was measured at 290 nm by using UV-VIS spectrophotometer. Blank was prepared without adding substrate. The PAL activity was expressed as total PAL activity (U/ml) in fresh seed weight. One enzyme unit is defined as the change in 0.01 unit of absorbance per minute.

2.1.2.2 Estimation of total crude protein in crude seed extract

The total protein content in the crude aril extract of Kandhari Kabuli was determined using Lowry method (Lowry *et al*., 1951) [20]. Intensity of the color developed was measured at 660 nm against the reagent blank. The amount of protein was calculated from standard curve prepared using bovine serum albumin (100 mg/ml).

2.2 Screening of phytochemicals in different varieties of *Punica granatum* L.

Aril extract of pomegranate varieties were screened for the presence of different phytochemicals *viz*., phenolic, flavonoid content and carbohydrate content. The experiments regarding phytochemical studies were carried out in the Department of Biotechnology, College of Horticulture and Forestry, Neri, Hamirpur. All the chemicals used for phytochemical estimation was procured from Himedia and Merck Pvt.Ltd (India).

2.2.1 Preparation of extract for phytochemical analysis:

Arils from the mature fruits of pomegranate varieties were manually separated. Arils (10 mg) were crushed with help of pestle and mortar to make a crude aril extract. This crude juice extract was dissolved in 10 ml of methanol. Aqueous extract of aril from pomegranate varieties were screened for estimation of total phenolic, flavonoid and carbohydrate content.

2.2.1.1 Estimation of total phenolic content in different varieties of *Punica granatum* L.

Total phenolic content was estimated by using Folin-Ciocalteau method (EIfalleh *et al*., 2012). The absorbance of sample was read after intense blue colour development through UV-VIS spectrophotometer (Thermo scientific) at 750 nm. Gallic acid was used as reference standard for making standard curve. Total phenolic content was determined using linear equation of standard curve prepared

with different concentration of gallic acid (0.2, 0.4, 0.6, 0.8 and 1.0). The content of total phenolic compound was expressed as mg of gallic acid equivalent (GAE mg/g of fresh tissue).

2.2.1.2 Estimation of total flavonoid content: During present studies total flavonoid content was estimated by using colorimetric aluminium chloride method (ElFalleh *et al*., 2012) [13]. The absorbance of sample was read after pale yellow colour development on UV-VIS spectrophotometer (Thermoscientific) at 510 nm. Quercetin was used as reference standard for making standard curve. The total flavonoid content was determined using linear equation of standard curve prepared with different concentration of quercetin (0.1, 0.2, 0.3, 0.4 and 0.5) The total flavonoid content was expressed as mg of quercetin per gram fresh tissue (QE mg/ g of fresh tissue).

2.2.1.3 Estimation of total carbohydrate content

Total carbohydrate content in the methanolic aril extract of pomegranate varieties was estimated by using DNS method given by Kaur *et al*., 2018 [17]. Absorbance of the sample was recorded at 540 nm. Dextrose was used as reference standard for making standard curve. The carbohydrate content was expressed as mg of dextrose (DE mg/g of fresh tissue).

2.3 Statistical analysis

Experiments regarding studies biochemical and phytochemical were set up in a completely randomized block (CRD) design (Cochran and Cox, 1963 and Gomez and Gomez, 1984) [15] and each experiment was performed in replicates. The data were analyzed using one-way and two way analysis of variance (ANOVA). Differences at $P<0.05$ were considered significant. The statistical analysis was carried out by using MS-Excel and OPSTAT. All the data expressed as mean ± SE (Standard error).

3. Results and Discussion

3.1 Screening of PAL acitivity in arils of different varieties of *Punica granatum* L

Ten different varieties of *P. granatum* were screened for presence of total PAL activity. Among all varieties, significantly highest PAL activity was determined in the aril extract of Phulle Super Bhagwa (158.56 ± 0.50 U/ml) and lowest PAL activity was found in Kandhari Kabuli (57.94 ± 0.12 U/ml). The results were significant at 5% level of significance and are presented in Table 1. The results reported during this study showed the highest value of PAL activity in soft seeded variety (Phulle super bhagwa). Similar results were observed in the studies of Zarei *et al*., 2016 [38]. They reported highest PAL activity in soft seeded pomegranate variety as compared to the hard seeded varieties. Similarly variations for PAL activity among different plants was observed by Seda *et al.*, in 2016 [32]. They reported that highest PAL activity (64.9 ± 0.1 U/mg) was found in Cyathabasis fructiculosa as compared to other plants Bambusa oldhamii (0.19 U/mg), Cucurbita pepo (26.6 U/mg) and Glycine max (0.43 U/mg).

Table 1: Screening of PAL activity in different varieties of *Punica granatum* L.

S. No	Varieties	Total PAL activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
1	Kandhari Hansi	73.02±0.68	9.08±0.02	8.04±0.08
2	Kandhari Kabuli	57.94±0.12	8.85±0.01	6.54±0.01
3	Mridula	92.02±0.28	9.53±0.02	9.65±0.02
4	P-26	67.76±0.19	9.03±0.01	7.50±0.01
5	Jodhpur Red	107.28±0.53	9.17±0.01	11.69±0.06
6	Nabha	78.94±0.27	9.02±0.01	8.75±0.04
7	G-137	139.60±0.30	9.76±0.01	14.29±0.03
8	Chawla	63.96±0.57	8.84±0.01	7.22±0.06
9	Wonderful	91.64±0.42	9.32±0.02	9.82±0.07
10	Phulle Super Bhagwa	158.56±0.50	9.92±0.01	15.98±0.05
C.D.		1.15	0.05	0.144

Data is represents the mean ± standard error of twelve values.

3.2 Estimation of total phenolic content in different varieties of pomegranate

During present studies total phenolic content was determined in the ten pomegranate varieties. Among all varieties, highest phenolic content was determined in methanolic aril extract of Phulle Super Bhagwa (14.22±0.05GAE mg/g fresh aril weight). The lowest phenolic content was determined in methanolic aril extract of Mridula (2.96±0.72 GAE mg/g fresh aril weight). The results were significant at 5% level of significance and are presented in Table 2. These results were found to be correlated with the results of other's studies. Elifallah *et al.*, in 2012 reported 11.84 ± 1.92 mg GAE/ g dry weight of total phenolic content in methanolic extract of pomegranate seeds. Sudha *et al*., in 2012 [33] studied total phenolic content in the fruits of pepino. They reported maximum total phenolic content in ripe fruit (14.44±0.042 mg GAE/g) and minimum phenolic content in unripe fruit (13.99±0.50 mg GAE/g) of pepino. Derakhshani *et al*., in 2018 [10] studied total phenolic and flavonoid content in pomegranate peel, juice and seed. They reported highest content of phenolic (413±16.84 mg GAE/g) in peel of pomegranate and lowest phenolic content (12.4±5.21mg GAE/g) was determined in the juice of pomegranate.

Table 2: Quantification of photochemical in aril extract of different varieties of pomegranate

S. No	Varieties	Total phenolic content (GAE mg/g fresh aril weight)	Total flavonoid content (Q E mg/g fresh aril weight)	Total carbohydrate content (DE mg/g fresh aril weight)
1	Mridula	2.96±0.72	8.05±0.03	32.52±0.38
2	G-137	5.08±0.08	7.73±0.03	18.19±0.41
3	Jodhpur Red	7.52±0.06	6.57±0.06	24.27±0.05
4	Phulle Super Bhagwa	14.22±0.05	6.28±0.07	31.02±0.07
5	P-26	7.93±0.12	8.29±0.06	25.77±0.07
6	Kandhari Hansi	6.66±0.10	6.37±0.07	33.77±0.03
7	Wonderful	6.82±0.13	8.31±0.07	18.85±0.12
8	Chawla	9.05±0.13	6.07±0.05	37.83±0.02
9	Nabha	6.65±0.10	6.39±0.05	30.91±0.39
10	Kandhari Kabuli	11.0±0.08	12.93±0.06	38.95±0.35
C.D.0.05	0.296		0.184	0.539

*Data represents the mean ±standard error of ten values.

3.3 Estimation of Total flavonoid content (TFC) in different pomegranate varieties

The total flavonoid content (12.93±0.06QE mg/g fresh aril weight) was found to be significantly highest in methanolic aril extract of Kandhari Kabuli. The lowest flavonoid content (6.07±0.05QE mg/g fresh aril weight) was determined in Chawla. The results were significant at 5% level of significance and are presented in Table 2. Elfallehe *et al*., in 2012 [13] monitored total flavonoid content in seed, flower, peel and leaves of pomegranate. They reported that flavoniod content was found to be highest (21.45 ± 0.58RE mg/g dry weight) in aqueous flower extract and lowest (3.30 RE mg/g dry weight) in the aqueous seed extract. Sudha *et al*., in 2012 [33] determined total flavonoid content in the pepino fruits. They reported that mature fruits of pepino was found to have 23.62±0.61 mg RE/g of flavonoid content. Derakhshani *et al*., in 2018 [10] studied total phenolic and flavonoid content in pomegranate peel, juice and seed. They reported highest content of flavonoid (54±8.96 mg rutin/g) in peel of pomegranate and lowest flavonoid content (1.8±1.03 mg rutin/g) in the juice of pomegranate.

3.4 Estimation of total carbohydrate content

In methanolic extract of aril the significantly highest carbohydrate content was observed in Kandhari Kabuli (38.95±0.35 DE mg/g fresh aril weight) and lowest carbohydrate content was observed in G-137 (18.19±0.41 DE mg/g fresh aril weight). The results were significant at 5% level of significance and are presented in Table 2. Dadashil *et al*., in 2013 [8] studied on biochemical composition and physicochemical characteristics of pomegranate. They reported that highest carbohydrate content (33.41±2.02) was found in cultivar Shavar and lowest (24.09± 1.26) in Pust sefid cultivar of pomegranate.

4. Conclusion

During present investigation the results of phytochemical screening in ten varieties of pomegranate showed that highest phenolic content was observed in Phulle Super Bhagwa (soft seeded). The total flavonoid and carbohydrate content was found to be maximum in Kandhari Kabuli (hard seeded). Hence, there is need to use chromatographic techniques for the extraction of these bioactive metabolites from the sourced pomegranate variety

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