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Ethno medicine, antioxidant potential and inter-specific variations of *Punica* L. species growing in Swat district, KP, Pakistan

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Abstract
The present study has endeavored to highlight the medicinal importance and free radical scavenging activity as well as genetic relationships among *Punica* species growing in Swat Valley, KP, Pakistan. Folin-Ciocalteu method revealed higher phenolic content of both species (*P. protopunica* and *P. granatum*), values as (81.540±0.831 and 84.048±0.702 µg/mL) respectively, while flavonoids were detected lower except for *P. protopunica* specie (88.503±1.321 and 79.711±1.417 µg/mL). Both *Punica* L. species (*P. protopunica* and *P. granatum*) displayed strong antioxidant activities, ABTS activities of *P. protopunica* ranged (ABTS, 92.13±0.33 to 66.62±1.87 µg/mL) While for *P. granatum* (94.49±0.06 to 63.68±0.98) and DPPH value for *P. protopunica* (92.89±0.87 to 68.03±1.12 µg/mL), while for *P. granatum* (91.12±0.32 to 68.01±0.85) respectively. When compared to the control (Ascorbic acid 96.32±0.90 for ABTS and 97.77±0.28µg/mL for DPPH). The intra and inter specific diversity among the 40 genotypes of *Punica* species viz., 20 genotypes of *P. granatum*, 20 of *P. protopunica*, were tested using morphometric and biochemical profiling. Twenty four morphological characters were counted for the assessment of intra and inter genetic variation through traits similarity index and cluster analysis. Morphologically, the two species were 53.846% similar. Total seed protein profiling was carried out on 12% slab gel electrophoresis; 10 bands were recorded in both *P. protopunica* and *P. granatum* with molecular weight ranging from 15KDa to 180KDa. Intra locus contribution toward the genetic disagreement was 10% in *P. protopunica* and 50% in *P. granatum*. In the same way, inter species locus contribution toward genetic diversity was 50%. Interestingly, locus 6, 7, 8, 9 and 10 (B/L-6-B/L-10) were monomorphic in the collected germplasms and may be shown as generic specific loci for *Punica* species. SDS-PAGE profiling based on two-way cluster plotting successfully determined the two species into separate clusters and the diversity found between and within *Punica* species may offer unique avenues for breeding and novel drug discovery.

Keywords: *Punica* L, Ethnomedicinal uses, Antioxidant potential, Inter-specific diversity, SDS-PAGE

Introduction
The genus *Punica* L. currently identified as nature's power fruit, are plants used in traditional medicine for the treatment of various ailments and fruit as well. It is the unique genus of family Punicaceae Horan. This genus is represented by two species viz., *P. granatum* (2n = 2x = 16) (Bennett and Leitch 2005) and *P. protopunica* Balf. f. (2n = 2x = 14) (Levin 2006; Youssef *et al*., 2017; Teixeira da Silva *et al*. 2013). There is consideration regarding the center of origin of the *Punica* L. (Adhikari and Adhikari, 2010); however, it is commonly stated that *P. granatum* possibly originated in Iran and from there spread to remaining parts of the world (Hajiahmadi *et al*. 2013) [13]. The wild pomegranate (*P. protopunica*) mainly grows in Georgia, Armenia, Azerbaijan and Central Asia from Iran-Afghanistan, and Turkmenistan to Pakistan (Rana *et al*. 2007) [32]. *Punica* species has high adaptive ability, as a crop, pomegranate is widely grown in tropical and subtropical regions. The main secondary center for *Punica* species is Mediterranean basin diversification mainly in countries, comprising of Albania, Montenegro, Tunisia, Morocco, Spain, Turkey, Egypt (Levin, 2006) [18].

The forests of *Punica* L. species consists of important natural communities in the subtropical dry temperate areas of the northern areas of Pakistan (Irshad *et al*., 2016). These *Punica* L. species have delivered various types of ecological and economic facilities to the local populations over decades (Khan *et al*., 2011) [16]. The Punica is notorious for its edible fruits with fleshy red, pink, or whitish external layers (Lama *et al*., 2001). It is a source of wood for fire, construction, and medicinal products to different ethnic groups from ancient times till present (Hazrat *et al*., 2007) [14].

Pomegranate is one of the richest dietary sources of antioxidant phenolics and anthocyanins, exhibiting a wide variation among genotypes in terms of these secondary metabolites (Ozgen *et al*. 2008) [28]. Anthocyanins are the major pigments responsible for the pomegranate fruit skin and aril colour. The regulation of anthocyanin biosynthesis during pomegranate fruit development has been investigated (Ben-Simhon *et al*. 2011) [7]. PgWD40 expression in the pomegranate fruit skin is required to regulate the expression of downstream structural genes involved in the anthocyanin biosynthesis. In addition, *Punica protopunica* exhibits anticancer activity. As one of the Yemeni plants used in traditional medicine, *P. protopunica* extracts displayed a pronounced cytotoxic effect against four cancer cell lines (Mothana *et al*. 2007). Moreover, *P. protopunica* exhibited relevant antiplasmodial, antileishmanial, or/and antitrypanosomal activity (Mothana *et al*. 2012) [23].

On the other hand, *P. protopunica* is endemic to the Yemeni island of Socotra of the Arabian Peninsula (Balfour 1888) [6], and is considered an ancestral species by Shilikina (1973) [33]. This *P. protopunica* illustrates several

morphological changes compared with *P. granatum*, such as larger and narrower leaves, diverse foliage, smaller flower and fruit size, evergreen, continuous flowering, white seeds (Al Shawish *et al*. 2006) [4]. *P. protopunica* was once thought as an endangered species (Plant Red Data Book 1978), however, current assessment suggests it is a vulnerable species with fragmented population (Miller and Morris 2004); struggles to conserve these species are required because these are the only congeneric species of *Punica*. A wide genetic base is critical to adaptation and will determine the future harshness of climate change influences. Genetic bottlenecks jeopardize the prospective of crop species for sustainable cultivation and render them vulnerable to stresses (Muhammad *et al*., 2018) [24].

The aim of conservation genetics is to uphold genetic diversity at various levels and to offer tools for plant population monitoring and assessment that can be applied for conservation strategies. Every plant species is genetically distinct by nature. Conservation struggles and related research are infrequently directed towards individuals but genetic variation is always measured in individuals and this can only be estimated by collections of plant genotypes in a population/species. This is only possible to recognize the genetic variation from phenotypic variation either by quantitative traits or qualitative traits but phenotypic traits are badly affected by environmental fluctuations, which thereby complicate the analysis of inherited variation (Muhammad, 2018) [24]. For the estimation of genetic diversity, various molecular approaches are applied such as biochemical evaluation at protein level and DNA based practices have advantages over the classical morphological techniques (Muhammad *et al*., 2018b) [26] but biochemical assessment at protein level is very inexpensive as compared with the molecular examination (Win *et al*., 2011). Amongst biochemical procedures, Sodium dodecyl sulphate polyacrylamide gel electrophoresis is a simple, consistent and cheap (Muhammad *et al*., 2018a) [25].

The present work aims to focus on the ethno medicinal uses, free radical scavenging activity and genetic association between these two species of genus *Punica* and evaluates the inter and intra genetic diversity in *P. granatum* and *P. protopunica* genotypes growing in the area.

Materials and Methods

Plant collection, questionnaire and data collection

In the present study several exploratory trips were arranged to different agro-ecological zones of Swat in 2017 – 2018. During expedition, of different zones i.e. Gat koto, Dagay, Qabarshah, Kota, Terang, Dool, Chargo Tangay, Swegalai, Faqir Abad, Zarkhela, Zawra, Gora, Chongai, Sharif Abad, Landakay, Kota, Aboha, Thana, Swegalai, Dadahara, Ziarat, Amlook Gharai, Shamra, Soray, Tangai Chena, Qalagay, Sarkhanai, Yakhtangay, Dokat, Qambo, Kasai, Soray, Landai Shah, Sarkhazano, Kasai, Sarbala, Teghak, Banjo Banda were selected for collection of samples the two species of the genus *Punica* L. for antioxidant potential, morphological characterization and estimation of genetic diversity in seed storage protein profile Specimens for each specie have been collected and processed using standard herbarium techniques (Alexiades, 1996) [5]. Ninety local informants were interviewed. The study was conducted from Febuary till December, 2017. The study was based on direct communication with the local informants of the area. A survey was established to interview local people concerning the medicinal values of plants. On the basis of this information, the medicinal value of study area's plants was noted. The methodology was adopted by following the work of Qureshi *et al*. (2009) [30]; Qureshi *et al*. (2008) [31] and Ahmad *et al*. (2009) [9].

The specimens were identified referring several Floras, viz., Flora of Pakistan, Hooker (1872-1897). The updated nomenclature of the identified species followed Siddiqui *et al*. (2007) [34] and Ahmed *et al*. (2009). Ethnomedicinal data has been collected through Participatory Rural Appraisal (PRA), which is based on communication with indigenous people and direct observation on the ground (Martin, 1995) [20]. The data have been recorded through semi-structured interviews with populaces involved in the plants management (Alexiades, 1996) [5].

Quantitative Analysis

Used value (UVi): Used Value index is a quantitative method that assesses the relative importance of each medicinal plant species based on their relative use among informers (Phillips, 1994) [29] and it was calculated by using the formula:

$$UVi = \sum Ui/N$$

Where; Ui= each informant cited number of use reports for a given medicinal plant species where, Ni is the total number of informants interviewed for a given medicinal plant species.

Relative frequency of citations (RFCs): To evaluate the traditional value and medicinal importance of each plant species in an area, the Relative Frequency of Citations formula was used (Tardio and Pardo-de-Santayana, 2008).

$$RFCs= FCs/N$$

Where; FCs = Number of local informants who reported traditional medicinal use of the species and N is the total number of informants of the study. (In this study, N = 90)

Consensus index (CI %): Percentage of local informants having traditional knowledge of plant species medicinal use against diseases (in this study) was calculated by Consensus index (CI %) which indicating citation by % of informants:

$$CI= n/N\times100$$

Where, n is the number of informants citing medicinal plant species, while N is the total number of respondents of the study.

Fidelity level (FL) value: The fidelity level (FL) is the percentage of indigenous informants claiming the given plant's use report for the same major ailment. It was calculated by the following formula (Alexiades, 1996) [5].

$$FL= Ip/Iu\times100$$

Where; Ip = Use of plant species suggested by the number of informants for a particular disease and Iu is the total number of informants who cited same plant for any disease.

Informant consensus factor (FIC): Informant consensus factor (FIC) value was used to analyze the consensus

between uses of Plants for various ailment categories and respondents of the study area; it was calculated by the following formula (Bhat *et al*., 2013) [8].

FIC= Nur- Nt/ Nur-1

Where, Nur = Number of use citations for a particular disease category while Nt is the number of botanical species used for a particular use category by all informants, the values of FIC range from 0 to 1. High value specifies that the informants are in favor on the use of plant species for a disease category and low value shows that plant species are randomly selected / informants do not exchange their traditional medicinal use knowledge.

Total Phenolic Content (TPC)

The total phenolic content of *Punica* L. species i.e. *Punica protopunica* and *P. granatum* (wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) these species were determined, following the method reported previously (Shirazi *et al*., 2014). In this assay 100µL of diluted extract was taken in a test tube, 500µL of distilled water and 100µL of Folin-Ciocalteu reagent were added, mixed and left for 6 min. Then 1000µL of 7% Sodium Carbonate and again 500µL of distilled water were added, respectively. After 90 min, absorbance was measured at 760nm using UV-Spectrophotometer. Gallic acid standard curve was obtained using the dilutions (31.05, 62.5, 125, 250, 500 and 1000 µg/ml, respectively) for measuring the TPC and was expressed as mg of Gallic acid equivalent per gram (mg GAE/g) of dry sample.

Total Flavonoid Contents (TFC)

Total flavonoid content of *Punica protopunica* and *P. granatum* species (wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) The fruits of these species were calculated using the previously reported procedure (Kim *et al*., 2003). Quercetin was used as a standard and TFC was determined as milligram of Quercetin equivalent (mg QE/g) per gram of dry sample. Calibration curve for Quercetin was obtained using a series of dilution (31.05, 62.5, 125, 250, 500 and 1000 µg/ml, respectively) prepared in methanol. 100µL each of these dilutions were mixed with 500µL of distilled water and 100µL of 5% Sodium nitrate was mixed and allowed for 6 min. After that 150µL of 10% aluminum chloride solution was added and allowed for 5 min. Finally 200 µL of 1M sodium hydroxide was added and absorbance recorded at 510 nm. The same procedure was repeated three times for all plants extracts.

DPPH free scavenging assay

The DPPH assay was carried out for *Punica protopunica* and *P. granatum* species (wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) crude methanolic extract following previously described technique (Brand-Williams, 1995). DPPH (2 mg) was dissolved in 100 ml of methanol to get DPPH solution. The stock solutions of samples having concentrations of 1 mg/ml were prepared in methanol and diluted to the concentrations of 1000, 500, 250, 125, 62.5 µg/ml, respectively. Diluted solution of 0.1 ml was taken from each sample mixed with 3 ml of DPPH solution in methanol. The solution was incubated at 23°C for 30 min and the absorbance was measured at 517 nm. For positive control ascorbic acid was used. Each concentration was taken in triplicate and the data obtained was presented as mean ± S.E.M. The percent radical scavenging activity was calculated using the following equation:

Percent DPPH scavenging activity=

$$\frac{\text{Control absorbance} - \text{Sampleabsorbance}}{\text{Controlabsorbace}} \text{x100}$$

ABTS free scavenging assay

Antioxidant potentials of *Punica protopunica* and *P. granatum* species (wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) The crude methanolic extract against 2, 2-azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) followed standard assay (Re-Pellegrini, 1999). Solutions of ABTS (7 mM) and potassium persulfate (2.45 mM) were prepared. The solution was kept overnight in dark to produce free radicals and absorbance of ABTS solution was adjusted to 0.7 at 745 nm by the addition of 50% methanol. Then 300 µl of samples were taken and 3 ml of ABTS solution was added to it and absorbance was measured at 745 nm for 6 min. For positive control Ascorbic acid was used. The data was recorded in triplicate and percent ABTS free radicals scavenging potential was calculated as follows:

Percent ABTS scavenging activity =

$$\frac{\text{Control absorbace} - \text{sample absorbace}}{\text{absorbace Control}} \text{ x 100}$$

Morphological characterization

For morphological data analysis both the qualitative and quantitative traits were taken. Quantitative traits which were measured with the help of vernier calipers are: petiole length (PL), leaf length (LL), leaf width (LW), Flower length (FL), Fruit length (Fl) seed length (SL), seed width (SW). Characters mean was found out after measuring of 3 different samples (small, medium, large) of each quantitative trait.

The observed qualitative characters are leaf shape (LS), leaf color (LC), seed texture (St), Hilum color (Hc), seed coat color (SC), seed shape (SS), leaf pubescent (LP), flower color (FC), Fruit shape (FS) and Fruit color (FtC). The data of both quantitative and qualitative characters of 40 genotypes (total 13 characters) was recorded and the binary matrix data was subjected to computer software the PCORD and the cluster analysis was presented as in Figure 1.

Protein Profiling

To assess genetic diversity and genetic relationships, SDS-PAGE was conducted (Lameli, 1970). For seed storage protein profile, single seed of each gnotypes was crushed into a powder. 400µl of Protein Extraction Buffer PEB (0.5M Tris-HCL, 0.2%SDS, 5M Urea, 1%B-mercaptoethanol under 8-pH) was added to 0.01g of seed powder. Then mixture in E-tube was vortexed thoroughly to homogenize it. The Comassie Brilliant Blue (CBB) was added to the E-tube as tracking dye to see the movement of PEB-FP on the separation PAG. The homogenated samples were centrifuged at 13,000 rpm for 10 minutes under room temperature. The electrophoretic process was carried out using 12% polyacrylamide gel (composition of resolution gel: 3.0M Tris-HCl pH9.0, 0.4% SDS and staking gel 0.4M Tris-HCl pH 7.0, 0.4% SDS). The electrode buffer

containing 0.025M Tris, 129M Glycine and 0.125% SDS was poured in the Electrophoresis tank. Similarly, 15µl PEB-FP was loaded in each well of 15% PAG. The electrophoresis was run at 100V until the blue line passed through the bottom of gel plates. The PAG were than stained and destained for data scoring of seed storage protein profile.

Results

Medicinal uses of various parts of two species of *Punica* L. growing in Swat

In this study 2 plant species of *Punica* L. (*P. protopunica* and *P. granatum*) belonging to Punicaceae family in Swat Valley, have been in recorded for curing of 12 categories of diseases. For each species botanical name, family, local name, ailments to be treated, and part(s) used were noted (Table1).

Quantitative trait analysis

Used Value (UVi), relative frequency of citations (RFCs), fidelity level (FL), consensus index (CI %)

Dominated medicinal plant between these two species, with most use values was *P. granatum* having UVi of 0.393 while 0.281for *Punica protopunica*. Based on the RFC values, the most cited medicinal plant species by the traditional drivers are *Punica protopunica* (RFCs= 0.731), while the second one was *Punica granatum* (0.551). The medicinal plant species with highest fidelity level was of *P. granatum* cited 75% for Mouth & throat sore, Skin diseases, High Blood pressure, Jaundice, Earache respectively. Percentage of respondents questioned with traditional medicinal knowledge about plant species used to treat ailments and Consensus index (CI) of the botanical taxa are mentioned in Table 1, which ranged from 50.61% to 73.03%. CI results revealed that most respondents percentage was for *Punica granatum* L. (CI%= 73.03%), followed by *Punica protopunica* (50.61%). Most consensus index for for some medicinal plant like *Berberis lycium* was also reported by Khan and Ahmad, (2015) [16]. CI indicates consensus on the importance of *Punica granatum* L. as important, well known medicinal plants used in cultural folk medicines and treat a number of disorders in the Swat valley. Similarly for different plants, results were found by Khan and Ahmad (2015) [16] who reported that 'CI' values are highest for *Berberis lyceum* Table 1.

Informant consensus factor (Fic)

Informant consensus factor (FIC) ranged from 0.972 to 1.000 (Table 2) and their use reports (URs) from 28 to 90. Different diseases *viz.* Anthelminthic, Anti-diabetics, Diarrhea, Digestive Problems, Dry Cough, Dysentery, Earache, High blood pressure Urinary infections had maximum FIC value due to representation by only single medicinal plant taxa/ species (*N*t = 1). Other most frequent and cited disease categories were Jaundice (FIC= 0.979), followed by Mouth & throat sore (0.977), Skin disease (0.972). Many disease categories having most citations or maximum number of species but their FIC values vary due to the basic indices of FIC formula as the consensus of informants on the use of medicinal plants against different disorders is described by the computed index FIC. Lowest FIC value was found in Skin diseases (0.972). Many researchers reported Anthelminthic, Anti-diabetics, Diarrhea, Digestive Problems, Dry Cough, Dysentery, Earache, High blood pressure Urinary infections as the most cited disease categories (Muhammad *et al*., 2018; Tangjitman *et al*., 2015) [24, 38]

Table 1: Medicinal plants with local and family name, habit, part used, disease cured, UVi, RFCs, FL, CI

S. No	Botanical Name	Local name	Family	Habit	Part used	Uvi	RFCs	FL%	CI%	Ailments
1	*Punica protopunica*	Anangoray	Punicaceae	Tree	Fruits peels, Seed, flower	0.281	0.731	74.358	50.61	Urinary infections, Dry Cough, Digestive Problems, Diarrhea, Skin disease, Anthelminthic, Anti-diabetics, Dysentery, Mouth & throat sore, Jaundice
2	*Punica granatum* L.	Anar	Punicaceae	Shrub	Root, fruits, leaves	0.393	0.551	75	73.03	Mouth & throat sore, Skin diseases, High Blood pressure, Jaundice, Earache

Table 2: Groups of disorders and informant consensus factor (ICF) for each category.

Disease categories	No. of Taxa (Nt) Used	Use Reports (Nur)	Fic
Anthelminthic	1	90	1.000
Anti-diabetics	1	87	1.000
Diarrhea	1	85	1.000
Digestive Problems	1	80	1.000
Dry Cough	1	78	1.000
Dysentery	1	75	1.000
Earache	1	62	1.000
High blood pressure	1	58	1.000
Jaundice	2	50	0.979
Mouth & throat sore	2	46	0.977
Skin disease	2	38	0.972
Urinary infections	1	28	1.000

Determination of (TPC) and (TFC)

The current studies of total phenolic/flavonoid content in different genotypes of *Punica protopunica* and *P. granatum* species (wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) was determined in methanolic extracts. Gallic acid was used as a standard to determine total flavonoid content and standard gallic acid curve was constructed by preparing the dilutions 20, 40, 60, 80, and 100 mg/ml, respectively. Results shows that the total phenolic contents of methanolic extract of wild *Punica protopunica* Pp-1 and Pp-2 were 84.048±0.702 and 78.032±0.884 and for *P. granatum* Pg-21 and Pg-22 was as 69.430±0.654 and 81.540±0.831mg respectively of gallic acid equivalent per gram (mg GAE/g) of dry sample. The highest TPC was shown by sample wild *Punica protopunica* Pp-1 (Table 3).

Quercetin was used as a standard for determination of total flavonoid contents. For this purpose a standard Quercetin curve was constructed by preparing the dilutions 20, 40, 60, 80 and 100 mg/ml. Results showed that the total flavonoid contents of methanolic extracts of wild *Punica protopunica* Pp-1 and Pp-2 were 87.691±1.452 and 88.503±1.321 and cultivated *P. granatum* Pg-21 and Pg-22 as 78.053±2.072and 79.711±1.417mg respectively of Quercetin equivalent per gram (mg QE/g) of dry sample respectively (Table 1), highest TFC was shown by sample *Punica protopunica* Pp-2 followed by samples *Punica protopunica* Pp-1.

DPPH scavenging assay

DPPH free radical scavenging potential was determined for four genotypes of *Punica* L. viz. *P. protopunica* and *P. granatum* species. (wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) methanolic extracts. Wild *Punica protopunica* Pp-1 and Pp-2 has shown 62.56±1.55 and 68.03±1.12 and for cultivated *P. granatum* Pg-21 and Pg-22 data ware recorded as 68.01±0.85 and 62.12±1.07 percent DPPH scavenging potential at lowest concentration range of 31.25 µg/ml, respectively. Results indicated highest %DPPH inhibition in genotype wild *Punica protopunica* Pp-2. The IC_{50} values of all genotype are shown in (Table 4). Ascorbic acid was used as a positive control and showed concentration dependent response. Ascorbic acid has shown 70.20±0.67% inhibitions at lowest concentration range of 31.25 µg/ml against DPPH with IC_{50} value 4.21µg/ml (Table 4).

ABTS scavenging assay

Free radical scavenging potential was also determined against ABTS of 4 genotypes of *Punica* L. *Punica protopunica* and *P. granatum* species. (Wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) methanolic extracts. Genotypes wild as (Wild *Punica protopunica* Pp-1 and Pp-2 while cultivated *P. granatum* Pg-21 and Pg-22) methanolic extracts have shown % ABTS inhibition of Pp-1 and Pp-2 61.68±0.98, 66.62±1.87 and cultivated *P. granatum* Pg-21 and Pg-22 as 63.68±0.98 and 62.62±0.97 respectively, at lowest concentration range of 31.25 mg/ml (Table. 4). Genotype *P. granatum* Pg-21 showed highest %ABTS inhibition value of 94.49±0.06mg/ml due to the presence of high phenolic compounds while, the %ABTS inhibition and IC_{50} values of all the genotype are shown (Table 4). Ascorbic acid was used as a positive control and has shown concentration dependent response of 69.56±0.93mg/ml at lowest concentration of 31.25 µg/ml against ABTS with IC_{50} value 5.14µg/ml (Table. 4).

Table 3: Represented the TPC (Total phenolic content) and TFC (Total Flavonoids content) in methanolic extracts of *Punica* L. (Wild and Cultivated) genotypes collected from different region of Swat

Samples	TPC (mg GAE/g)	TFC(mg QE/g)
Wild *Punica protopunica* Pp-1	84.048±0.702	87.691±1.452
Wild *Punica protopunica* Pp-2	78.032±0.884	88.503±1.321
P. granatum Pg-21	69.430±0.654	78.053±2.072
P. granatum Pg-22	81.540±0.831	79.711±1.417

Table 4: ABTS and DPPH radical scavenging activity of *Punica* L. genotypes at different concentration

S.NO	Genotypes	Concentration (µg/ml)	%ABTS Scavenging Mean ± SEM	IC50	%DPPH Scavenging Mean ± SEM	IC 50
1	Wild *Punica protopunica* Pp-1	1000	86.49±0.16	9.07	90.05±1.12	8.76
		500	83.56±0.36		85.43±0.76	
		250	78.29±0.91		80.31±0.44	
		125	71.46±1.38		76.29±0.71	
		62.5	67.79±0.80		68.48±0.53	
		31.25	61.68±0.98		62.56±1.55	
2	Wild *Punica protopunica* Pp-2	1000	92.13±0.33	10.35	92.89±0.87	7.89
		500	85.21±0.70		88.28±0.67	
		250	82.94±0.69		81.00±0.89	
		125	78.67±0.59		76.96±1.01	
		62.5	70.89±0.62		70.70±0.65	
		31.25	66.62±1.87		68.03±1.12	
3	*P. granatum* Pg-21	1000	94.49±0.06	9.90	91.12±0.32	8.89
		500	86.76±0.96		88.54±0.56	
		250	79.39±0.81		84.89±0.77	
		125	70.49±0.78		79.27±0.76	
		62.5	68.79±0.80		74.49±0.53	
		31.25	63.68±0.98		68.01±0.85	
4	*P. granatum* Pg-22	1000	80.13±0.44	10.32	90.01±0.89	8.34
		500	83.21±0.89		87.78±0.54	
		250	79.94±0.89		80.11±0.67	
		125	76.67±0.69		78.43±1.03	
		62.5	69.89±0.90		73.45±0.64	
		31.25	62.62±0.97		62.12±1.07	
5	Ascorbic acid	1000	96.32±0.90	5.14	97.77±0.28	4.21
		500	89.45±0.74		92.57±0.82	
		250	83.89±0.66		87.26±0.37	
		125	78.57±0.46		81.10±0.47	
		62.5	73.51±0.81		75.35±0.76	
		31.25	69.56±0.93		70.20±0.67	

Genetic association among the genotypes of two species of *Punica* L. based on morphological and Biochemical characterization

Morphological Characterization

Data matrix of 40 genotypes based on morphology was analyzed for the construction of phylogenetic tree to represents the similarity of these two species of the *Punica* L. and the phylogenetic tree was constructed (fig 1). The phylogenetic tree divided the two species in two lineages/ Regions R-I and R-II. The R-II holds the 20 genotypes of *P. granatum* while R-II enclosed all genotypes of *P. protopunica*. The similarity indexes were performed for all the genotype of 2 species that was 53.846% for *P. protopunica* and *P. granatum* (Table 5).

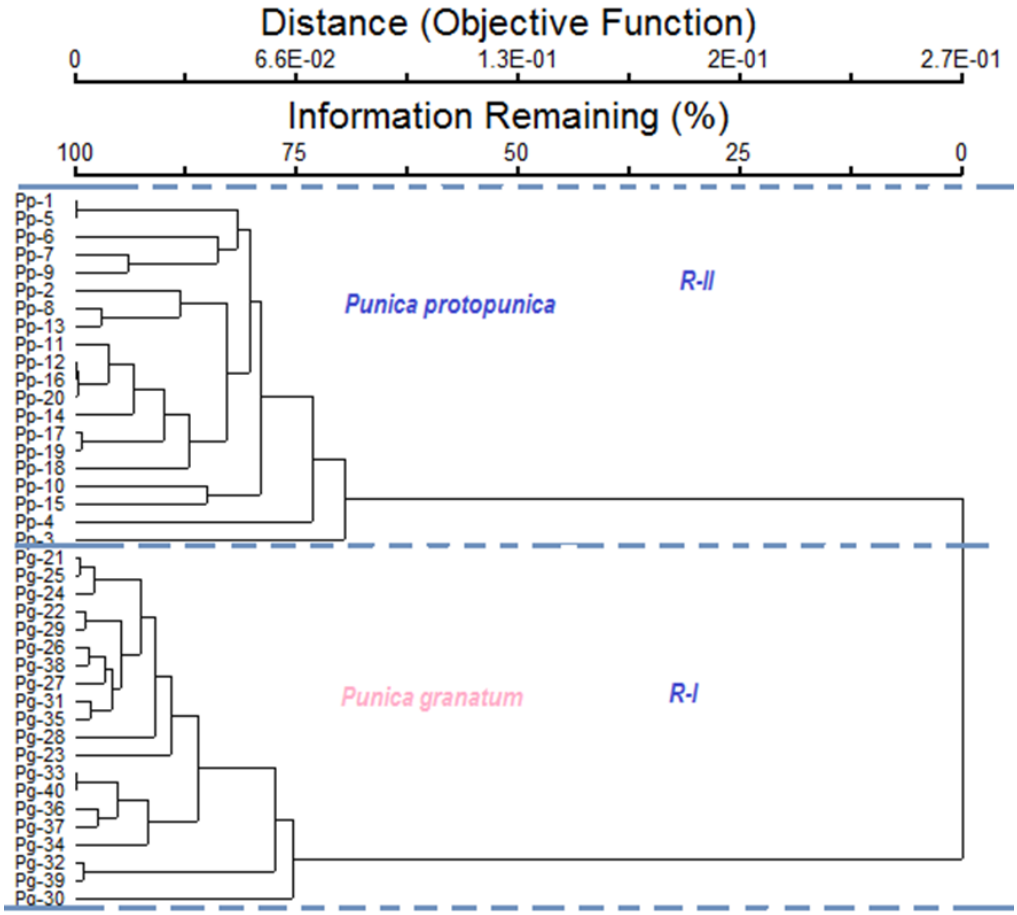

Fig 1: Phylogenetic tree based on morphological data showing diversity of two species of *Punica* (*P. protopunica* and *P. granatum)*. Pp= *Punica protopunica*, Pg= *Punica granatum.*

Table 5: Intra and interspecific genetic diversity in 24 morphological characters studied in P. protopunica and P. granatum.

Traits	*P. protopunica* L.	*P. granatum* L.	Trait similarity index P.p & P.g
PL	3.5	4	NA
LL	6.5	7.12	NA
LW	3.21	3.45	3.33
FL	3	3.35	*3.175
Fl	6.8	7.065	NA
SL	*3.9333	*5.663	NA
SW	2.333	4.8	NA
LS	**Oblong-lanceolate**	**Oblong-lanceolate**	**Oblong-lanceolate**
LC	*Dark green	*Dark green	*Dark green
LP	*Present	*Present	*Present
FC	Pink	Red	NA
FS	*Globose	*Globose	*Globose
FtC	*Brownish	*Brownish	*Brownish

Total TSI = 53.846% ((homologous trait/total traits)*100)

*- Traits similarity within two species

PL= Petiole length, LL= Leaf length, LW= Leaf width, FL= Flower length, Fl= Fruit length, SL= Seed length, SW=Seed width, LS= Leaf Shape, LC= leaf Color, Leaf pubescent, FC= Flower color, FS= Fruit Shape, FtC= Fruit Color, P. p= *P. protopunica*, *P. granatum*

Biochemical characterization

Ten protein bands were detected in the *Punica* L. species (*P. protopunica* and *P. granatum*) (Suppl: Figure). The phylogenetic relationship between all the genotypes of two species through phylogenetic tree has been revealed in the (Figure 2). The dendrogram divided into two regions (R-I and R-II) at linkage distance 0.05. The region I (R- I) was composed of all the 20 genotypes of *P. granatum* (collected from *P. granatum*-29 Gat koto, *P. granatum*-28 Dagay, *P. granatum*-27 Qabarshah, *P. granatum*-26 Kota, *P. granatum*-25 Terang, *P. granatum*-24 Dool, *P. granatum*-23 Chargo Tangay, *P. granatum*-22 Swegalai, *P. granatum*-23 Faqir Abad, *P. granatum*-22 Zarkhela, *P. granatum*-21 Melagah, *P. granatum*-20 Zawra, *P. granatum*-40 Gora Gat, *P. granatum*-39 Chongai, *P. granatum*-38 Sharif Abad, *P. granatum*-37 Landakay, *P. granatum*-38 Kota, *P. granatum*-36 Aboha, *P. granatum*-35 Thana, *P. granatum*-34 Swegalai, *P. granatum*-33 Dadahara, *P. granatum*-32 Ziarat,

P. granatum-31 Amlook Gharai, *P. granatum*-30 Shamra while the region II (R-II) was consisted of the all genotypes of *P. protopunica* (collected from *P. protopunica*- 1 Soray, *P. protopunica*-2 Tangai Chena, *P. protopunica*- 3 Qalagay, *P. protopunica*- 4 Sarkhanai, *P. protopunica*- 5 Yakhtangay, *P. protopunica*- 6 Dokat, *P. protopunica*- 7 Qambo, *P. protopunica*- 8 Kasai, *P. protopunica*- 9 Soray, *P. protopunica*- 10 Tangai Chena, *P. protopunica*- 11 Landai Shah, *P. protopunica*- 12 Sarkhazano, *P. protopunica*- 13 Dokat, *P. protopunica*- 14 Sarkhanai, *P. protopunica*- 15 Dokat, *P. protopunica*- 16 Qalagay, *P. protopunica*- 17 Yakhtantangay, *P. protopunica*- 18 Qambo, *P. protopunica*- 19 Kasai, *P. protopunica*- 20 Sarbala, (Figure 2). Decisively, the R-I and R-II grouped 20 genotypes each of *Punica* L.

Genetic disagreement at protein level

Remarkably, Table 6 shows interspecific variation among 40 genotypes of the *Punica* L. species. Among all the genotypes10 loci (L1-L10) were noted out of these B/L-6, 7, 8, 9 and L10 were monomorphic and were marked as generic specific which is used to classify the *Punica* L. species. Furthermore, the loci B/L-1, B/L-2, B/L-4 and B/L-5 were marked as polymorphic with 20%, 77.50%, 92.50%, 22.50% and 22.50% genetic diversity, respectively. The inter species comparative locus contribution toward genetic disagreement (CLCTGD) was 50% of the two species of 40 *Punica* L. genotypes (Table 6).

Table 7 represents the intraspecific variation among the 20 genotypes of *P. protopunica*, exhibited low intra-specific locus variation. Among 10 loci, out of which B/L-1, B/L-4 B/L-5, B/L-6, B/L-7, B/L-8, and B/L-9 and in B/L-10 were monomorphic, while B/L-2 was polymorphic. The B/L-3 was missing in 20 *P. protopunica* genotypes hence this locus can be helpful to identify this specie. B/L-2 represents 60 percent variation. The locus contribution toward genetic disagreement (LCTGD) of *P. protopunica* was 10% (Table 7).

Intraspecific locus variation among 20 genotypes of *P. granatum* is represented in Table 8 and 10 loci/ bands. Notably, B/L-6, B/L-5, B/L-6, B/L-7, B/L-8, B/L-9 and B/L-10 were monomorphic in *P. granatum* while B/L-1, B/L-2, B/L-3, B/L-4 and B/L-5 were polymorphic and showed 50, 55%, 85%, 45%, 45% variation and the locus contribution toward genetic disagreement (LCTGD) of *P. granatum* was 50%.

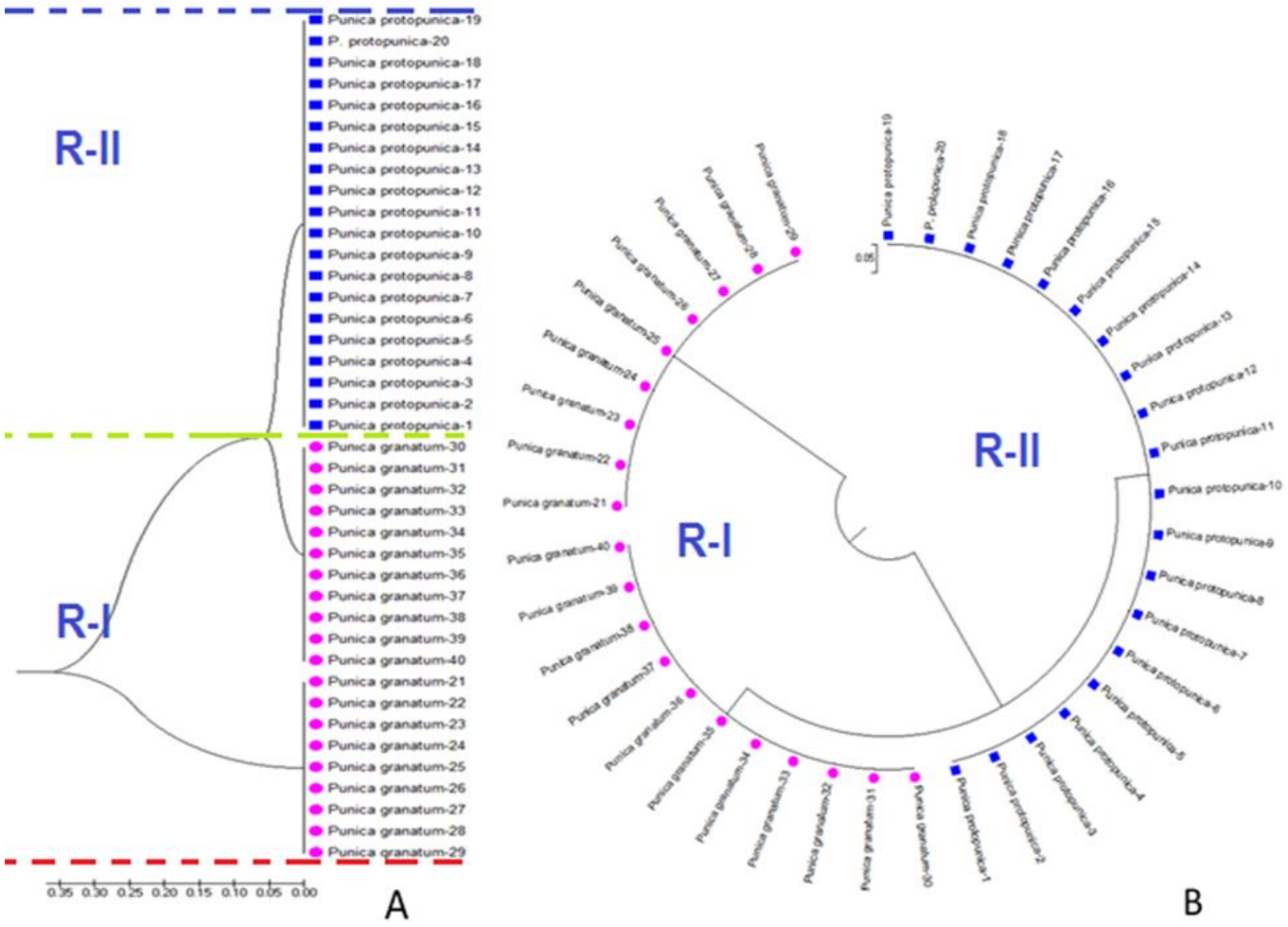

Fig 2: Inter -species phylogenetic relationship identified through SDS_PAGE analysis in 40 different genotypes of *Punica* species collected from Swat, Khyber Pakhtunkhwa, Pakistan.

Table 6: Inter specific diversity among the genotypes of *P. proptpunica* and *P. granatum*

Locus	Present (%)	Absent (%)	Variaton	Status	GD
B/L-1	32(80%)	8(20%)	20%	Poly	0.8
B/L-2	9(22.5%)	31(77.5)	77.50%	Poly	0.45
B/L-3	3(7.5%)	37(92.5%)	92.50%	Poly	0.075
B/L-4	31(77.5%)	9(22.5%)	22.50%	Poly	0.775
B/L-5	31(77.5%)	9(22.5%)	22.50%	Poly	0.775
B/L-6	40(100%)	0.00	Nil	Mono	1.00
B/L-7	40(100%)	0.00	Nil	Mono	1.00
B/L-8	40(100%)	0.00	Nil	Mono	1.00
B/L-9	40(100%)	0.00	Nil	Mono	1.00
B/L-10	40(100%)	0.00	Nil	Mono	1.00

GD=50% (GD= Poly loci/Total loci*100)
GB= Genetic diversity, B/ L= Bands/ locus

Table 7: Intra specific diversity among the genotypes of *P. proptpunica*

Locus	Present %	Absent %	Variation	Status	GD
B/L-1	20(100%)	0.00	Nil	Mono	1.00
B/L-2	8(40%)	12(60%)	60%	Poly	0.4
B/L-3	0.00	20(100%)	Nil	Mono	0.00
B/L-4	20(100%)	0.00	Nil	Mono	1.00
B/L-5	20(100%)	0.00	Nil	Mono	1.00
B/L-6	20(100%)	0.00	Nil	Mono	1.00
B/L-7	20(100%)	0.00	Nil	Mono	1.00
B/L-8	20(100%)	0.00	Nil	Mono	1.00
B/L-9	20(100%)	0.00	Nil	Mono	1.00
B/L-10	20(100%)	0.00	Nil	Mono	1.00

GD=10% GD= Poly loci/Total loci*100)
GD= Genetic diversity, B/L= Bands/ locus

Table 8: Inter specific diversity among the genotypes of P. granatum

Locus	Present %	Absent %	Variation	Status	GD
B/L-1	10(50%)	10(50%)	50	Poly	0.5
B/L-2	9(45%)	11(55%)	55%	Poly	0.45
B/L-3	3(15%)	17(85%)	85%	Poly	0.15
B/L-4	11(55%)	9(45%)	45%	Poly	0.55
B/L-5	11(55%)	9(45%)	45%	Poly	0.55
B/L-6	20(100%)	0.00	Nil	Mono	1.00
B/L-7	20(100%)	0.00	Nil	Mono	1.00
B/L-8	20(100%)	0.00	Nil	Mono	1.00
B/L-9	20(100%)	0.00	Nil	Mono	1.00
B/L-10	20(100%)	0.00	Nil	Mono	1.00

GD=50% GD= Poly loci/Total loci*100)
GD= Genetic diversity, B/L= Bands/ locus

Discussion

This study is the first to use ethno medicinal uses, antioxidant potential and biochemical markers. The frequently used medicinal plant between these two species, with most used value was *P. granatum* having (UVi= 0.393) while the lowest value was found for *Punica protopunica* (0.281). Maximum used values of cited medicinal plant species might be due to their extensive distribution and cultural driver's awareness which resulted these botanical species as the first choice for treatment (Ullah *et al*., 2014). Those plants reported by the interviewees were directed for all the local populaces of the research area. Based on the RFC values, the most cited medicinal plant species by the traditional drivers are *Punica protopunica* (RFCs= 0.731), while the second one was *Punica granatum* (0.551). Maximum relative frequency citations clarify the facts that these medicinal plant species are very well known among the most number of traditional drivers (Butt *et al*., 2013). Those medicinal plant species having maximum RFC should be further evaluated phytochemically and pharmaceutically to identify their active constituents for drug discovery (Vitalini *et al*., 2013). The medicinal plant species with highest fidelity level was of *P. granatum* cited 75% for Mouth & throat sore, Skin diseases, High Blood pressure, Jaundice, Earache respectively. These mentioned plant species may be confirmed as important medicinal plants through further assessment and evaluation through phytochemical, biological and pharmaceutical activities. Many researchers obtained maximum fidelity level values against certain disorders (Lulekal *et al*., 2013) [19]. Moreover, plants with minimum FL should not be abandoned as declining to remark them to the future generation that it could raise the threat of gradual depletion of the cultural knowledge (Chaudhary *et al*., 2006) [10]. CI results revealed that most respondents percentage was for *Punica granatum* L. (CI%= 73.03%), followed by *Punica protopunica* (50.61%). Most consensus index for for some medicinal plant like Berberis lycium was also reported by Khan and Ahmad, (2015) [16]. CI indicates consensus on the importance of *Punica granatum* L. as important, well known medicinal plants used in cultural folk medicines and treat a number of disorders in the Swat valley. Similarly for different plants, results were found by Khan and Ahmad (2015) [16] who reported that 'CI' values are highest for Berberis lycium. Different diseases viz. Anthelminthic, Anti-diabetics, Diarrhea, Digestive Problems, Dry Cough, Dysentery, Earache, High blood pressure Urinary infections had maximum FIC value due to representation by only single medicinal plant taxa/ species (Nt = 1). Folin-Ciocalteu method revealed higher phenolic content of both species (*P. protopunica* and *P. granatum*), values as (81.540±0.831 and 84.048±0.702 µg/mL) respectively, while flavonoids were detected lower except for *P. protopunica* specie (88.503±1.321 and 79.711±1.417 µg/mL). Both *Punica* L. species (*P. protopunica* and *P. granatum*) displayed strong antioxidant activities, ABTS and DPPH activities of *P. protopunica* ranged (ABTS, 92.13±0.33 to 66.62±1.87 µg/mL) While for *P. granatum* (94.49±0.06 to 63.68±0.98) and DPPH value for *P. protopunica* (92.89±0.87 to 68.03±1.12 µg/mL), while for *P. granatum* (91.12±0.32 to 68.01±0.85) respectively. When compared to the control (Ascorbic acid 96.32±0.90 for ABTS and 97.77±0.28µg/mL for DPPH).

The phylogenetic relationship and to measure the genetic relationship between *Punica protopunica* and *P. granatum* and to evaluate the genetic diversity among and within collected genotypes of *P. granatum* and *P. protopunica* genotypes were collected from various geographical regions in Swat. Our results provided sufficient information to aid in understanding the genetic relationship and diversity in *Punica* species. In all genotypes the SDS-PAG markers

were informative given that they exhibited almost low intra and high inter genetic diversity and the total Genetic disagreement (%GD) generated by this systems were high. Various techniques such SRAP has been used previously for genetic diversity assessment in *P. granatum* by Soleimani *et al*. (2012) [36]. Other studies indicated that TRAP and ITAP are useful in plant genotyping and genetic diversity assessment (Xiong *et al*. 2013) [41].

The inter-specific diversity between two species was 50% in *Punica* species genotypes. The locus contribution toward genetic disagreement (LCTGD) of *P. granatum* and *P. protopunica* was 10% and 50% respectively. Due to intra-specie locus contribution toward genetic variation SDS-PAGE could be a dependable technique for documentation of these species (Muhammad *et al*., 2018b) [26]. The genetic pool of dissimilarity within genotypes of this specie is the basis for selection as well as for plant improvement and conservation (Muhammad *et al*., 2018) [24]. A better knowledge of genetic diversity and its distribution in the genotypes of the studied plants species is vital for its conservation and application. This will aid significantly in describing what to conserve as well as where to conserve, and will enhance our information and understanding of the taxonomy, origin and evolution of *Punica* L**.**

Conclusions

This work has attempted to high point the medicinal importance, antioxidant potential as well as genetic relations among *Punica* species growing in Swat Valley, KP, and Pakistan. The medicinal plant species with highest fidelity level was of *P. granatum* cited 75% for Mouth & throat sore, Skin diseases, High Blood pressure, Jaundice, Earache respectively. Informant consensus factor (FIC) ranged from 0.972 to 1.000 and their use reports (URs) from 28 to 90. Different diseases *viz.* Anthelminthic, Anti-diabetics, Diarrhea, Digestive Problems, Dry Cough, Dysentery, Earache, High blood pressure Urinary infections had maximum FIC value due to representation by only single medicinal plant taxa/ species (*N*t = 1). Folin-Ciocalteu method revealed higher phenolic content of both species (*P. protopunica* and *P. granatum*), values as (81.540±0.831and 84.048±0.702 µg/mL) respectively, while flavonoids were detected lower except *P. protopunica* specie (88.503±1.321 and 79.711±1.417 µg/ml). Both *Punica* L. species (*P. protopunica* and *P. granatum*) displayed strong antioxidant activities, ABTS and DPPH activities of wild *P. protopunica* ranged (ABTS, 92.13±0.33to 66.62±1.87 µg/ml) While cultivated (94.49±0.06 to 63.68±0.98) and DPPH value for wild *P. protopunica* (92.89±0.87 to 68.03±1.12 µg/mL), while for cultivated (91.12±0.32 to68.01±0.85), respectively. When compared to the control (Ascorbic acid 96.32±0.90 for ABTS and for DPPH 97.77±0.28µg/ml). respectively. SDS profiling could be suitable tool for genotype identification and variability analysis (between and within *Punica*). The intra and inter specific diversity among the 40 genotypes of *Punica* species viz., 20 genotypes of *P. granatum*, 20 of *P. protopunica*, were tested using morphometric and biochemical profiling. Twenty four morphological characters were counted for the assessment of intra and inter genetic variation through traits similarity index and cluster analysis. Total seed protein profiling was carried out on 12% slab gel electrophoresis; 10 bands were recorded in both *P. protopunica* and *P. granatum* with molecular weight ranging from 15KDa to 180KDa. Intra locus contribution toward the genetic disagreement was 10% in *P. protopunica* and 50% in *P. granatum*. In the same way, inter species locus contribution toward genetic diversity was 50%. Interestingly, locus 6, 7, 8, 9 and 10 (B/L-6-B/L-10) were monomorphic in the collected germplasms and may be shown as generic specific loci for *Punica* species.

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Conflict of interest

The authors have no potential conflict of interest to declare.

Significance statement

There is a great potential in *Punica* L. as a medicinal plant/food crop particularly in the hilly areas of Swat Kp, Pakistan and requires national policy to support its cultivation and Conservation.

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