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Hexaploid-Tetraploid Landraces and Wild Species of Wheat Revealed Diversity for Antioxidants and Total Phenolics

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Wheat contains various phytochemicals, most importantly, antioxidants and polyphenolic compounds that have a variety of health-promoting effects. Seed material was obtained from 63 wheat genotypes of three species (*T. aestivum*, *T. durum*, and *T. sphaerococcum*) from Pakistan and Syria. This study was initiated to estimate and compare the interspecific and intraspecific diversity for antioxidant activity (AOA) and total phenolic content (TPC) in different species of wheat. The wild relatives and landraces of three wheat species exhibited a highly significant interspecific and intraspecific diversity for both traits. *T. aestivum* exhibited more intraspecific diversity. The AOA of the genotypes ranged from 7.45% to 41.68%, being maximum in accessions of *T. durum* 12977 (41.68%). TPC ranged from 10.09 to 39.28 mg GAE g^{-1} and was highest in *T. durum* accession 12999 (39.28 mg GAE g^{-1}). The lowest AOA (7.45%) and TPC (10.09 mg GAE g^{-1}) were observed in *T. aestivum* landrace LR-27. Hence, *T. durum* accessions 12999 and 12979 could be our desired accessions for future breeding programs having phytochemicals. Cluster analysis distributed the genotypes into four clusters. Genotypes of different origins grouped differently, indicating an environmental influence in the development of their genetic architecture. Cluster analysis indicated that 41.30% of the genotypes were included in the high AOA and TPC group. Members of *T. aestivum* that grouped in clusters 1 and 2 showed low to moderate AOA and TPC. The accessions of *T. durum* and *T. sphaerococcum* performed much better than *T. aestivum* for both of the biochemical traits.

Key Words: antioxidants, landraces, phytochemicals, total phenolics, *Triticum*, wheat

Abbreviations: AOA — antioxidant activity, TPC — total phenolic content, ROS — reactive oxygen species

INTRODUCTION

Wheat is cultivated throughout the world, and being a staple food, it is a major source of health-promoting components (Abozed et al. 2014; Laddomada et al. 2015; Tayyem et al. 2016). Its grains are also a vital source of various antioxidant compounds, for example, phenolic anthocyanins and phenolic acids that can scavenge free radicals to help in preventing chronic diseases such as neurodegenerative diseases (Eliasova and Paznocht 2017) and non-infectious diseases (Okarter and Liu 2010). The most important thing is the quantification of phytochemicals that are mostly present in whole-wheat grain and its products. Hence, the utilization of whole-wheat grain might be helpful to reduce the risk of several chronic ailments (Liu 2007).

Ferulic acid is one of the major phenolic compounds found in wheat, accounting for 57% to 77% (Zhou and Yu 2004). Wheat also contains hydroxyl-cinnamic acid derivatives that show antioxidative properties (Shahidi and Wanasundara 1992). Phenolics are also important for plant adaptation, even phenomena such as phototropism is accomplished via phenolic photo-receptors. The biosynthesis of phenolic substances is affected by environmental factors (Andreasen et al. 2001). The phytochemical response of whole grains varies with change in environment, location, altitude, genotype, and genetics of the plant (Guo et al. 2011; Disna et al. 2017).

Phytochemicals are more common in the endosperm and the aleuronic layer of kernel. They are vital for plant survival because plants face different stresses *in vivo* that lower their growth, productivity and yield by producing free radicals (Rao et al. 2013). Stress factors produce different reactive oxygen species (ROS) and reactive free

radicals, and phytochemicals can decrease the damage of oxidative stresses (Chandrasekara et al. 2012). Whole grains, vegetables and fruits are major sources of naturally occurring antioxidants. Although the increased consumption of whole grain cereals and whole grain-based products has been closely related to reduced risk of chronic diseases, bioactive compounds found in whole grain cereals have not achieved as much attention as the bioactive compounds in vegetables and fruits (Leváková and Lacko-Bartošová 2017).

Domestication and artificial selection has created homogeneity in several crop species. Breeders are now revisiting the sources of genetic diversity that were left unutilized during domestication and were now found as wild relatives, in addition to the primeval germplasm such as landraces (Acquaah 2012). However, there are some reports that different species of grain crop including wheat have a large diversity in terms of these bioactive compounds which can be exploited. Wheat grain and its bran extracts contain antioxidant properties (Yu et al. 2005). The nutritive value of many crops has been increased by transferring genes from wild relatives into the domesticated ones, for example, the nutritive value (vitamin C and beta carotene) and solids content has been increased in tomato which significantly enhanced its market value (Acquaah 2012). Thus, diversity of biochemical traits may need to be explored among the wild relatives and landraces for introgression of genes into cultivated varieties.

Although there are many analytical methods for measuring the antioxidant activity of food products such as wheat, different methods give different results due to use of a particular free radical as a reactant such as hydroxyl radical (OH), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. It has been widely used to assess the free radical scavenging capability of different grain crops (Kumar et al. 2011). The proton radical scavenging activity is an essential feature of antioxidants, assessed by DPPH radical scavenging method (Khurshid et al. 2018). Antioxidant molecules have a hydrogen-donating capacity that contributes to its radical scavenging nature. To assess the diversity of these vital bioactive compounds, cluster analysis can be used, which is effective to study the genetic variation and reveal the family relationship (Azim et al. 2018). Considering the benefits and importance of phytochemicals, this research work was initiated to estimate and compare the extent of diversity for antioxidants and total phenolics among hexaploid and tetraploid species of wheat.

MATERIALS AND METHODS

This research work was conducted in the Department of Plant Breeding and Molecular Genetics, University of Poonch Rawalakot, Azad Kashmir from 2016 to 2017.

Seed Material

The seed of 63 genotypes of diverse origin and genetic background including three different wheat species (*T. durum, T. aestivum* and *T. sphaerococcum*) were used to assess antioxidant activity (AOA) and total phenolic content (TPC) (Table 1). The seed of 24 Pakistani landraces was acquired from the Department of Plant Breeding and Genetics, PMAS-University of Arid Agriculture, Rawalpindi while those of 38 wild accessions of Pakistan and Syria and a check variety 'BARS-2009' were acquired from the National Gene Bank, Plant Genetic Resource Institute (PGRI) NARC, Islamabad. Since the grains were acquired from various institutes and were produced and stored under diverse environments, and to reduce the influence of different environmental conditions on the phytochemical properties of the grains, the material was sown in a randomized complete block design with three replications at the University of Poonch Rawalakot (latitude: 33°51'28.15"N, longitude: 73°45'37.55"E, elevation: 1737 m) under uniform conditions. Harvested grains of each genotype were bulked and used for further analysis.

Estimation of Phytochemicals

Dry and mature wheat grains were ground using mortar and pestle to obtain a fine powder. For the estimation of antioxidant activity, samples were prepared by taking 1 g whole grain flour of each genotype, added to 10 mL of 97% ethanol. Extraction was carried out with shaking at room temperature. Extracts were separated by filtering through filter paper (Whatman No. 1). The resulting residue was further extracted twice and the whole extract was concentrated in a rotary evaporator (50 °C). Serial dilutions were prepared to obtain the desired concentration of plant samples for the experiments (Sedej et al. 2010; Khan et al. 2014). Antioxidant activity was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as defined by Hatano et al. (1988). A 0.25 mM DPPH solution (0.5 mL) was added to 1 mL of ethanolic extracts at different concentrations (50–1,000 μg/mL) and kept in the dark for 30 min. Optical density (OD) of the samples was recorded with the use of a UV-VIS Spectrophotometer (Analytik Jena AG, Germany) at 517 nm for 100 mL concentration. The corresponding blank readings (control) were also taken for each sample. The antioxidant activity was expressed as percentage scavenging of DPPH radical by the extracts as follows:

Table 1. List of wheat germplasm containing three different species of wheat and their origin.

Sr. No.	Name/ Acc. No.	Species	Origin	Sr. No.	Name/ Acc. No.	Species	Origin
1	BARS-2009	*T. aestivum*	Pakistan	33	12983	*T. durum*	ICARDA-Syria
2	LR-3	*T. aestivum*	Pakistan	34	12984	*T. durum*	ICARDA-Syria
3	LR-5	*T. sphaerococcum*	Pakistan	35	12985	*T. durum*	ICARDA-Syria
4	LR-6	*T. aestivum*	Pakistan	36	12986	*T. durum*	ICARDA-Syria
5	LR-7	*T. aestivum*	Pakistan	37	12988	*T. durum*	ICARDA-Syria
6	LR-10	*T. aestivum*	Pakistan	38	12989	*T. durum*	ICARDA-Syria
7	LR-11	*T. aestivum*	Pakistan	39	12992	*T. durum*	ICARDA-Syria
8	LR-12	*T. aestivum*	Pakistan	40	12993	*T. durum*	ICARDA-Syria
9	LR-13	*T. aestivum*	Pakistan	41	12996	*T. durum*	ICARDA-Syria
10	LR-15	*T. aestivum*	Pakistan	42	12998	*T. durum*	ICARDA-Syria
11	LR-16	*T. aestivum*	Pakistan	43	12999	*T. durum*	ICARDA-Syria
12	LR-20	*T. aestivum*	Pakistan	44	13000	*T. durum*	ICARDA-Syria
13	LR-26	*T. aestivum*	Pakistan	45	13001	*T. durum*	ICARDA-Syria
14	LR-27	*T. aestivum*	Pakistan	46	13002	*T. durum*	ICARDA-Syria
15	LR-30	*T. aestivum*	Pakistan	47	13003	*T. durum*	ICARDA-Syria
16	LR-33	*T. aestivum*	Pakistan	48	13004	*T. durum*	ICARDA-Syria
17	LR-34	*T. aestivum*	Pakistan	49	13005	*T. durum*	ICARDA-Syria
18	LR-35	*T. aestivum*	Pakistan	50	13006	*T. durum*	ICARDA-Syria
19	LR-36	*T. aestivum*	Pakistan	51	13007	*T. durum*	ICARDA-Syria
20	LR-37	*T. aestivum*	Pakistan	52	13008	*T. durum*	ICARDA-Syria
21	LR-38	*T. aestivum*	Pakistan	53	13009	*T. durum*	ICARDA-Syria
22	LR-41	*T. aestivum*	Pakistan	54	13010	*T. durum*	ICARDA-Syria
23	LR-42	*T. aestivum*	Pakistan	55	13011	*T. durum*	ICARDA-Syria
24	LR-43	*T. aestivum*	Pakistan	56	13012	*T. durum*	ICARDA-Syria
25	LR-44	*T. aestivum*	Pakistan	57	13013	*T. durum*	ICARDA-Syria
26	12976	*T. durum*	ICARDA-Syria	58	13014	*T. durum*	ICARDA-Syria
27	12977	*T. durum*	ICARDA-Syria	59	13015	*T. durum*	ICARDA-Syria
28	12978	*T. durum*	ICARDA-Syria	60	13016	*T. durum*	ICARDA-Syria
29	12979	*T. durum*	ICARDA-Syria	61	19027	*T. sphaerococcum*	CRP-Wheat/NARC
30	12980	*T. durum*	ICARDA-Syria	62	19028	*T. sphaerococcum*	CRP-Wheat/NARC
31	12981	*T. durum*	ICARDA-Syria	63	19029	*T. sphaerococcum*	CRP-Wheat/NARC
32	12982	*T. durum*	ICARDA-Syria				

Scavenging = [(Absorbance of control − Absorbance of sample) x 100]/Absorbance of sample

The IC_{50} values (extract concentration that results in 50% scavenging) were determined from the graph of the scavenging effect percentage against the extract concentration.

The TPC was estimated via the Folin-Ciocalteau method (Ghafoor and Choi 2009). This method depends on the reduction of Folin's reagent by phenols to a mixture of blue oxides that has maximum absorption in the region of 765 nm. 200 µL of the diluted sample of different concentrations was mixed with 400 µL of Folin Ciocalteu reagent. Gallic acid (1 mg/mL) was used as control. For dilution of samples and controls, double distilled water was used, mixed and incubated at room temperature for 10 min. Then 1 mL of 20% Na_2CO_3 solution was added, mixed and kept for 2 h at room temperature. The absorbance was then recorded at 765 nm using a spectrophotometer. The total phenolic content of samples was expressed in milligram Gallic acid equivalent (GAE) per 100 mL (mg GAE/100 mL).

Statistical Analysis

Three readings were recorded for each sample, expressed as means and subjected to single-factor analysis of variance using MS-Excel (Snedecor and Cochran 1989). Non-hierarchical cluster analysis was performed based on the standard distance of k-means using computer software Statistica V 6.0 while hierarchical cluster analysis was done using Ward's method with the help of statistical

package PAST V. 3.20 (Hammer et al. 2001).

RESULTS AND DISCUSSION

Wheat (genus *Triticum*) is considered to be an important source of polyphenols, plant secondary metabolites with numerous health-promoting effects. Many phytochemicals are responsible for the high antioxidant activity of whole-grain products (Leváková and Lacko-Bartošová 2017).

Analysis of Variance and Mean Values

The analysis of variance indicated highly significant statistical differences between the genotypes and among the species for AOA and TPC (Table 2).

Wheat extracts indicated a strong scavenging activity against the free radicals generated by DPPH (Fig. 1 and 2). The genotypes showed an AOA in the range of 7.45–41.68%. The highest antioxidant activity was observed in *T. durum* accession 12977 (41.68%) closely followed by accessions 12999 (40.73%) and 12979 (40.51%) while the minimum was found in landrace LR-27 (7.45%). Abozed et al. (2014) recorded an antioxidant activity of 28.1–31.8% and 33.8–36.8% in wheat varieties Gemiza-9 and Beni-suef -3, respectively. When compared with the other two species, *T. durum* accessions performed significantly better in terms of antioxidant ability. Antioxidant properties displayed by these wheat genotypes can eliminate the free radicals produced in the body and reduce the oxidative damage. Yu et al. (2005) reported that wheat grains contain several bioactive compounds that can enhance their antioxidant properties.

The phenolics are found in wheat and other cereal grains in free and conjugated forms (Jinli et al. 2018). The mean values of TPC ranged from 10.09 to 39.28 mg GAE g^{-1} (Fig. 2 and 3). The genotypes found high in AOA were also high in TPC. Maximum TPC was again found in *T. durum* accession 12999 (39.28 mg GAE g^{-1}) closely followed by accessions 12977 (39.13 mg GAE g^{-1}) and 13003 (38.58 mg GAE g^{-1}) while the least was noted in *T. aestivum* landrace LR-27 (10.09 mg GAE g^{-1}). Wheat genotypes having more phenolic content can be identified as a source of antioxidants to produce functional foods (Yilmaz et al. 2018). Intake of food that can reduce oxidative damage to important biomolecules like proteins and DNA has been considered to prevent carcinogenesis as well as coronary heart diseases in humans (Zhou and Yu 2004; Delvalle 2011). Besides, the phenolics may have anti-mutagenic activities and decrease the risk of diabetes (Broekaert et al. 2011). It has already been demonstrated that wheat grains contained an optimum level of natural antioxidants (Aprodu and Banu 2012). The *T. durum* and *T. sphaerococcum* accessions contributed more towards TPC compared with the *T. aestivum* landraces. The relative performance of *T. aestivum* landraces in terms of AOA and TPC was poor in comparison with the other two species.

Cluster Analysis

Hierarchical clustering techniques are the most popular and widely used but they offer several disadvantages. Non-hierarchical methods, on the other hand, have also gained increased acceptability but they too have several shortcomings. As both techniques have merits and demerits, it is often suggested to use both methods in combination to confirm the results (Awan et al. 2015). Therefore, both techniques were applied in this study to benefit from the advantages of both.

Cluster analysis showed huge interspecific and intraspecific diversity in wheat. Based on k-mean clustering, the genotypes were divided into four main clusters (Table 3). Cluster 1 comprised 7 landraces of *T. aestivum* viz., LR-5, LR-20, LR-27, LR-30, LR-37, LR-38 and LR-41 which accounted for 11.10% of the total genotypes. The landraces included in this group were the lowest in antioxidant activity and total phenolics. In this cluster, the maximum value for AOA was shown by LR-5 (21.32%) and least by LR-27 (7.45%), while TPC was maximum in LR-37 (18.25 mg GAE g^{-1}) and least in LR-27 (10.09 mg GAE g^{-1}). The cluster 2 included 8 landraces of *T. aestivum* (LR-10, LR-11, LR-12, LR-13, LR-15, LR-33, LR-35, and LR-42) and 3 *T. durum* accessions (12996, 12998 and 13004). 17.5% of the total genotypes were included in this cluster, which were moderate in antioxidant activity and total phenolics. Minimum antioxidant activity was noted in landrace LR-13 (23.85%) while the lowest value for TPC was noted in landrace LR-12 (21.39 mg GAE g^{-1}).

Cluster 3 included 30.20% of the total genotypes (BARS-2009, LR-3, LR-6, LR-7, LR-16, LR-34, LR-36, 12982, 12983, 12989, 12992, 13000, 13001, 13006, 13007, 13008,

Table 2. Analysis of variance for antioxidant activity and total phenolic content in three species of wheat.

Source of Variation	Between Species	df	Within Species	df	F-value	F-tab
Antioxidant activity	519.50	2	33.97	60	15.29**	4.98
Total phenolic content	703.03	2	29.95	60	23.47**	4.98

**Significant at α = 0.01
df: degree of freedom, F-tab: F tabulated value

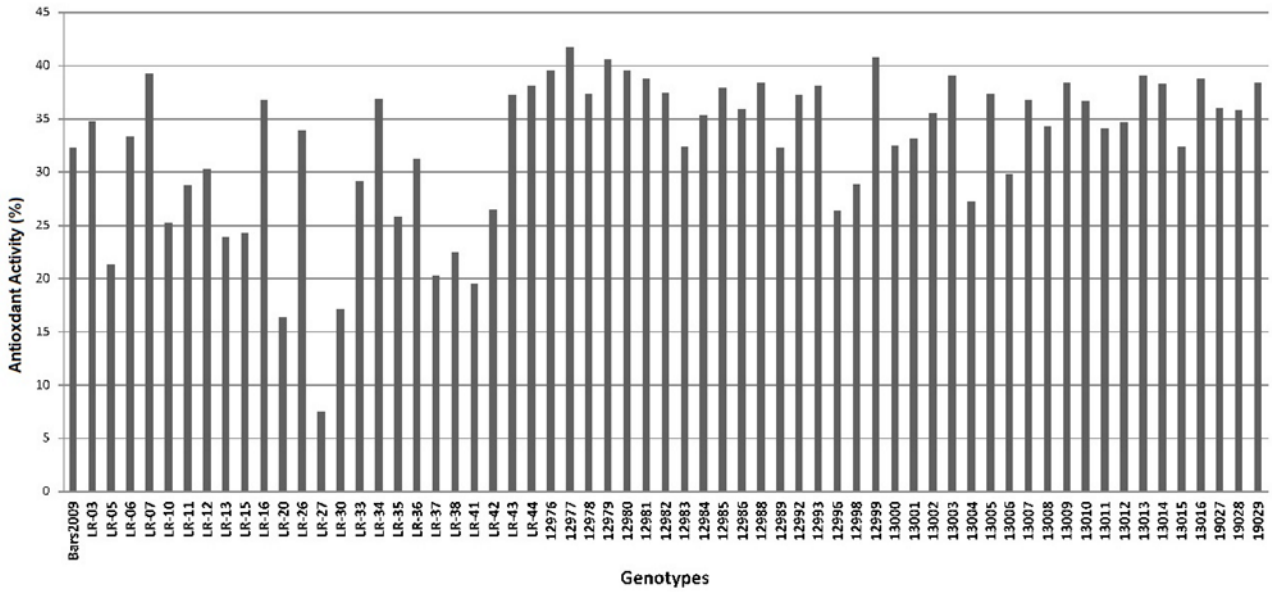

Fig. 1. Mean values for antioxidant activity in 63 broad-based wheat genotypes.

13011, 13015 and 19028). This group showed moderate to higher values for AOA and TPC. The AOA was maximum in *T. durum* accessions 12982 (37.39%) followed by 12992 (37.18%) and 13007. For TPC, *T. durum* accession 12983 added the most (32.41 mg GAE g^{-1}) followed by the check variety BARS-2009 (32.23 mg GAE g^{-1}). Higher AOA and TPC were depicted by the genotypes included in cluster 4 (LR-26, LR-43, LR-44, 12976, 12977, 12978, 12979, 12980, 12981, 12984, 12985, 12986, 12988, 12993, 12999, 13002, 13003, 13005, 13009, 13010, 13012, 13013, 13014, 13016, 19027 and 19029) accounting for 41.30% of the total genotypes. It was quite encouraging that almost 40% of the genotypes had higher AOA and TPC that could be utilized for further studies. The AOA was maximum in *T. durum* accessions 12977 (41.68%) followed by 12999 and 12979. The least value was noted in accession 12986 (35.87%) for AOA, while for TPC, the maximum value was found in accession 12999 (39.28 mg GAE g^{-1}) followed by 12977 and 13003; the minimum was found in 12984 (34.04 mg GAE g^{-1}).

Cluster analysis based on the hierarchical clustering method (Fig. 4) confirmed the results obtained from non-hierarchical clustering. Based on AOA and TPC, the genotypes of different origins and species grouped differently in various clusters. It seemed that the place of origin played a significant role in the development of the genetic architecture of genotypes, particularly the genes related to the antioxidant properties of wheat grains. The phylogenetic analysis conducted by Li et al. (2019) also showed that differences existed for polyphenol oxidase activity genes in plant materials from a different geographical origin in *T. monococcum* ssp. *aegilopoies*. Some studies have concluded that the content of phenolic acids is related to genotype in *Triticum spelta* (Hernández et al. 2011; Ragaee et al. 2012; Leváková and Lacko-Bartošová 2017). However, others have found that location (i.e., environmental factors) is more important (Vaher et al. 2010).

Pakistani landraces were mostly poor in AOA and TPC while *Triticum durum* accessions of Syrian origin were better in performance. Species also behaved differently in terms of their phytochemical constituents. The Pakistani origin wild accessions of *Triticum sphaerococcum* were also superior in antioxidant potential.

Table 3. Members of clusters based on k-mean clustering in 63 broad-based wheat genotypes.

Cluster No.	Members	Status of Antioxidant Activity and Total Phenolic Content	Names of Members
Cluster 1	07 (11.1%)	Low	LR-5, LR-20, LR-27, LR-30, LR-37, LR-38, LR-41
Cluster 2	11 (17.5%)	Moderate	LR-10, LR-11, LR-12, LR-13, LR-15, LR-33, LR-35, LR-42, 12996, 12998, 13004
Cluster 3	19 (30.2%)	Moderate to high	BARS-2009, LR-3, LR-6, LR-7, LR-16, LR-34, LR-36, 12982, 12983, 12989, 12992, 13000, 13001, 13006, 13007, 13008, 13011, 13015, 19028
Cluster 4	26 (41.3%)	High	LR-26, LR-43, LR-44, 12976, 12977, 12978, 12979, 12980, 12981, 12984, 12985, 12986, 12988, 12993, 12999, 13002, 13003, 13005, 13009, 13010, 13012, 13013, 13014, 13016, 19027, 19029

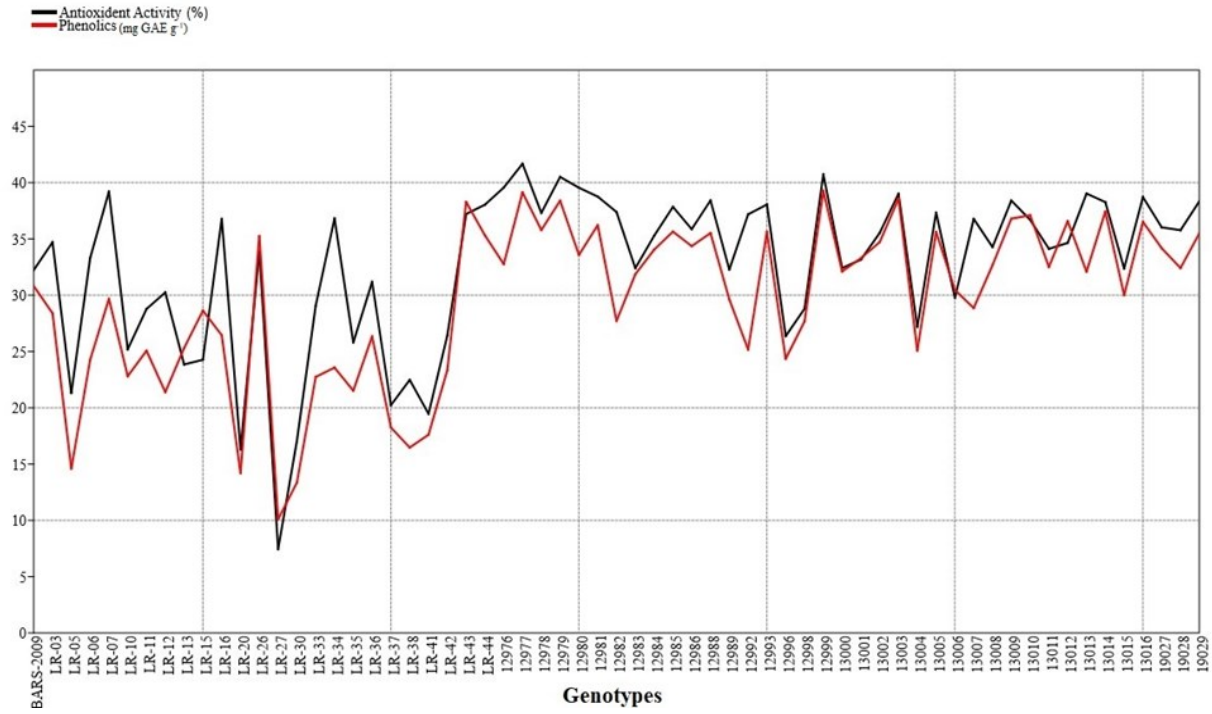

Fig. 2. Line diagram revealing diversity for antioxidant activity in wheat germplasm.

However, some landraces such as LR-26, LR-43, and LR-44 grouped in cluster 4 could be utilized in further breeding to enhance antioxidant properties in grains. *T. aestivum* showed comparatively more intraspecific diversity as it was distributed in all four clusters while the wild accessions of *T. durum* and *T. sphaerococcum* were grouped only in clusters having moderate to high AOA and TPC. There was maximum genetic distance between clusters 1 and 4 (20.56) (Table 5). Hence, members of these two clusters may be utilized as parents to initiate a breeding program aiming to increase AOA and TPC in wheat grain.

Comparison of wheat species (Table 4) indicated that the wild accessions of *T. durum* and *T. sphaerococcum* were superior in AOA and TPC compared with *T. aestivum* landraces. Therefore, whole-wheat grain consumption of these species may be recommended for a

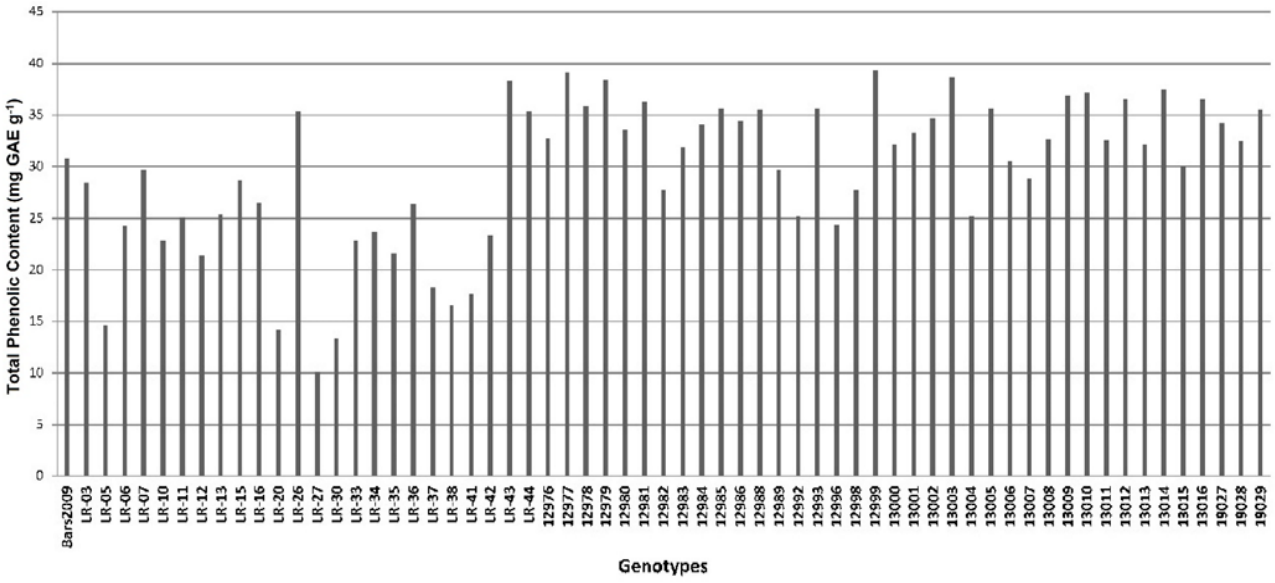

Fig. 3. Mean values for total phenolic content in wheat germplasm.

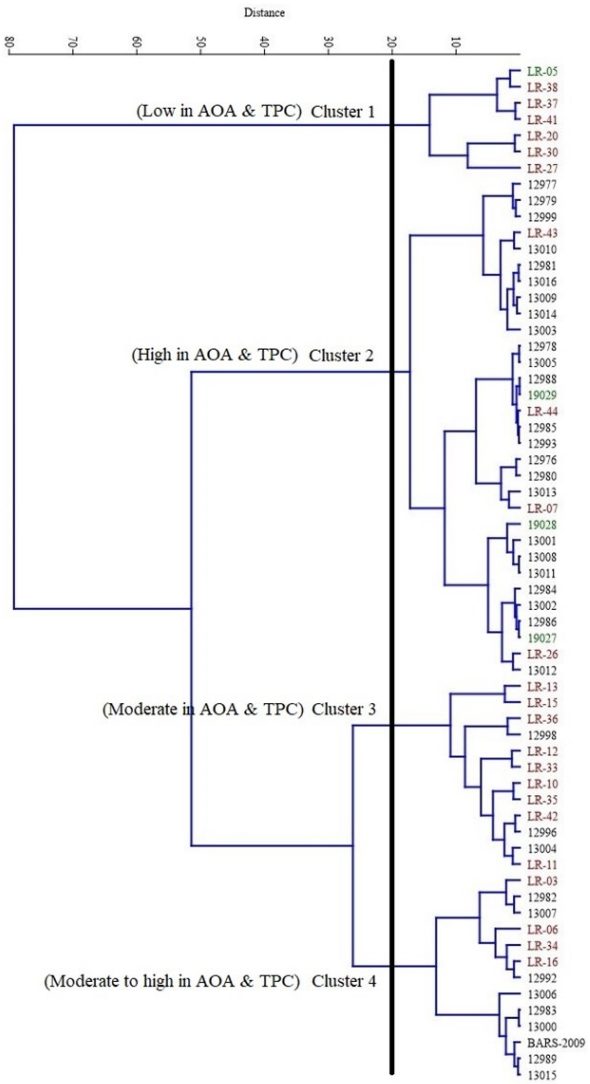

Fig. 4. Cluster diagram based on average linkage distance among 63 broad-based wheat genotypes.

healthy diet as indicated by Wood (2004). Similarly, Brandolini et al. (2013) reported that durum and bread wheats had the highest content of bound phenolic acids compared with other species of wheat. Li et al. (2008) recorded total phenolic acid content in *Triticum monococcum* similar to that in winter, spring and durum wheats, slightly higher than spelt, but marginally lower than emmer wheat.

The highest percentage of *T. aestivum* landraces were included in the groups having low to moderate (85.71% and 72.72%, respectively) AOA and TPC while the accessions of *T. durum* were included in the groups of moderately high to high (57.89 and 80.77%, respectively) AOA and TPC. Many cereal breeders are breeding

Table 4. Members of three wheat species in various quantitative groups based on antioxidant activity and total phenolic content.

Quantitative Group	*T. aestivum* (%)	*T. durum* (%)	*T. sphaerococcum* (%)
Low	85.71	00.00	14.29
Moderate	72.72	27.27	00.00
Moderate to high	36.84	57.89	05.26
High	11.54	80.77	07.69

Table 5. Euclidian distance among the clusters obtained from three species of wheat.

	Cluster 1	Cluster 2	Cluster 3	Cluster4
Cluster 1	0			
Cluster2	09.28	0		
Cluster 3	15.49	06.29	0	
Cluster 4	20.56	11.28	05.34	0

genotypes that may have maximum health benefits for our consumption (Jaafar et al. 2013; Ivanisova et al. 2014). Similarly, the variability existing in these genotypes can be exploited to breed new genotypes having maximum health benefits.

CONCLUSION

The ethanolic extracts of wheat grains displayed an optimum level of antioxidant potential and scavenging ability against DPPH radicals. The wild relatives and landraces of wheat exhibited a significant interspecific and intraspecific diversity in their scavenging abilities and total phenolic content. The phytochemical activities found in these genotypes can be exploited for introgression into cultivated varieties. The wild accessions of *T. durum* and *T. sphaerococcum* showed much better antioxidant properties and total phenolic content. The accessions 12999, 12977, 13003 and 12979 showed potential as functional food having antioxidant properties.

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