Antioxidant Activity and Phytochemical Content of Nine Amaranthus Species

Jun-Hyoung Bang 1,‡, Kyung Jun Lee 2,†, Won Tea Jeong 3, Sehee Han 1, Ick-Hyun Jo 4,§, Seong Ho Choi 5, Hyunwoo Cho 1,‡, Tae Kyung Hyun 1, Jeehye Sung 6, Junsoo Lee 7, Yoon-Sup So 8,* and Jong-Wook Chung 1,*‡

Abstract: Amaranthus species are widely used as grain and leaf vegetables around the world and are potential dietary sources of antioxidants and phenolic compounds. In this study, we examined the variation in total flavonoid contents, total polyphenol contents, and antioxidant activities among 120 accessions of nine Amaranthus species. The antioxidant activity of DPPH (2,2-diphenyl-1-picrylhydrazyl) of the 120 amaranth accessions ranged from 1.1 (A. tricolor) to 75.2 (A. tricolor) mg AAE/g in 2018, and 8.5 (A. tricolor) to 68.8 (A. dubius) mg AAE/g in 2019. ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate)) antioxidant activity ranged from 16.7 (A. tricolor) to 78.3 (A. hypochondriacus) mg AAE/g in 2018, and 36.6 (A. tricolor) to 54.7 (A. hypochondriacus) mg AAE/g in 2019. Total flavonoid content (TFC) of 2018 and 2019 ranged from 21.7 (A. hybridus) to 52.7 (A. hybridus) and from 22.3 (A. viridis) to 54.7 (A. tricolor), respectively. Antioxidant activities were compared using two methods and all components were measured in plants grown both in 2018 and 2019. We identified wide variation among the accessions and between plants grown in the two years. Antioxidant activities and phytochemical contents were consistently negatively correlated. The nine species and 120 accessions clustered into three groups according to their antioxidant activities, total flavonoid contents, and total polyphenol contents in each year. These results provide information about the nutritional profiles of different Amaranthus species.

Keywords: amaranth; annual; antioxidant activity; phytochemicals

1. Introduction

Amaranth or pigweed (Amaranthus spp.) is a gluten-free pseudocereal that belongs to the Amaranthaceae family and includes approximately 70 species. Members of this horticultural genus are mainly grown in Mexico and South America but thrive across the world, from cool-temperate to tropical regions [1,2]. The most commonly cultivated amaranth species are the grain species Amaranthus caudatus, Amaranthus cruentus, and Amaranthus

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*Correspondence: yoonsupso@chungbuk.ac.kr (Y.-S.S.); jwchung73@chungbuk.ac.kr (J.-W.C.)
† These authors have contributed equally to this work.
§ Tel.: +82-43-261-2510 (Y.-S.S.); +82-43-261-2518 (J.-W.C.)
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* and Jong-Wook Chung
The leafy amaranth species *Amaranthus hybridus* and *Amaranthus tricolor* are also cultivated, but to a lesser extent [3]. Amaranth is easily grown, adapts well to challenging growth environments, and has no major diseases [3]. Recently, Amaranthus extracts were reported to have antioxidant, antimalarial, and antiviral properties [4–8].

Developing robust, locally-adapted varieties of crops relies on knowledge both of the genetic variation among accessions and of the variation among genotypes in their sensitivity to the environment [9]. Phenotype is a function of the genotype, the environment, and the differential phenotypic responses of genotypes to different environments, also known as genotype by environment interactions [10]. Phytochemical content is affected not only by genetic variation, but also by environmental conditions and seasonal and year-to-year differences [11]. For instance, the phytochemical and antioxidant activity of 172 soybean (*Glycine max* L.) landraces differed between two cultivation years [7] and the phytochemical composition and biological activity of *Parkia speciosa* seeds varied significantly depending on where the plants had been cultivated [12].

A number of phytochemicals including flavonoids, alkaloids, tannins, phenolics, saponins, glycosides have been isolated from various *Amaranthus* sp. such as *A. caudatus*, *A. hypochondriacus*, and *A. cruentus* [7,13–15]. Lopez-Mejía et al. mentioned that it is very important to consider amaranth as potential antioxidant source due to tendencies for “new” natural ingredients demanded by consumers nowadays [4].

Reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, may contribute to cytotoxicity (e.g., chromosome aberrations, protein oxidation, and muscle injury) and to metabolic and morphologic changes (e.g., increased muscle proteolysis and changes in the central nervous system) in animals and humans [16]. Antioxidants are an important nutritional component of the human diet, as they prevent ROS from causing undesirable oxidative stress and thus protect against many human diseases. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are often added to processed foods, but as these compounds may have unwanted side effects, there is interest in identifying natural sources of antioxidants [16–18].

Although many methods of measuring antioxidant activity have been developed and used, they each have limitations and are difficult to compare because of varied reaction mechanisms and different phase locations [19]. Therefore, the relative antioxidant capacity index (RACI) was developed and proposed as a method to calculate and compare the antioxidant capacity of various samples [19]. The RACI method has been used to determine the antioxidant capacity of various species, such as orange (*Citrus sinensis* L.) [20], Astragalus gymnolobus, *A. leporinus* var. *hirsutus*, and *A. onobrychis* [21], soybean [11], and grapevine (*Vitis vinifera* L.) [22].

Various *Amaranthus* sp. have been evaluated for their antioxidant properties among which include *A. caudatus* [23,24], *A. cruentus* [24], *A. hypochondriacus* [4,24], *A. graecizan* [25], *A. viridis* [5,8,13], and *A. spinosus* [7,26]. Various methods such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate)) assays have been used to analyze the antioxidant activities of plant extracts, including various *Amaranthus* sp. [25,27–30]. Although these methods have given different results among tested samples and across laboratories, the results reported are significantly correlated with each other [31,32].

In this study, we aimed to determine the total phenolic content (TPC) and total flavonoid content (TFC) and to characterize the antioxidant activities of accessions from nine *Amaranthus* species. In addition, we analyzed variation between data obtained from plants grown in different years and correlations among TPC, TFC, and antioxidant activities. The results of this study provide useful information for amaranth breeding efforts aimed at producing nutritious food materials.
2. Materials and Methods

2.1. Plant Materials

One hundred and twenty accessions of nine *Amaranthus* species were obtained from the National Agro-biodiversity Center (NAS) of the Rural Development Administration (RDA), Republic of Korea (http://genebank.rda.go.kr, accessed on 4 March 21) (Table S1). These accessions were cultivated at the experimental field of Chungbuk National University, Korea in 2018 and 2019. For each accession, three seeds were sown in each well, and then thinned in the greenhouse to one plant per well after germination on 15 April 2018 and 23 April 2019, respectively. Two weeks after germination, transplanting was done to a field at CNU. A good plant stand was assured by transplanting individual *Amaranthus* seedlings every 20 cm within the furrow of these rows. Ninety days after transplanting, the leaves of each accession were collected and lyophilized using freeze dryer (FreeZone Freeze Dry System, Labconco, Kansas City, MO, USA).

2.2. Extractions from Leaves

One-hundred milligrams of ground sample was added to 1 mL of 75% EtOH and sonicated for 1 h. Then, the mixture was centrifuged at 16,873 × g for 10 min. The clear supernatant was collected in a new tube and used for the total phenolic content and antioxidant activity assays.

2.3. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Antioxidant Activity Assay

DPPH radical-scavenging activities of the extracts were assessed using the method of Lee et al. [33] with some modifications. DPPH solution (150 µL; 150 µM in anhydrous EtOH) was added to 100 µL of sample solution. The mixture was shaken vigorously and left to stand at 25 °C in the dark for 30 min. Absorbance at 517 nm was measured using a spectrophotometer (Epoch; Bio-Tek, Winooski, VT, USA). DPPH was reported as milligrams of ascorbic acid equivalents (AAE) per gram of dried weight sample (mg AAE/g dry leaf).

2.4. 2′-Azinobis (3-Ethylbenzothiazoline 6-Sulfonate) (ABTS) Antioxidant Activity Assay

ABTS radical-scavenging activity was estimated using the method of Lee et al. [33] with some modifications. The ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate, followed by an overnight incubation in the dark at room temperature. The ABTS radical cation solution was diluted with methanol (MeOH) to obtain an absorbance of 0.7 ± 0.02 at 735 nm. The diluted ABTS radical cation solution (190 µL) was added to 10 µL of sample solution. After 6 min, absorbance at 735 nm was determined using a spectrophotometer (Epoch; Bio-Tek, Winooski, VT, USA). DPPH was reported as milligrams of ascorbic acid equivalents (AAE) per gram dried weight sample (mg AAE/g dry leaf).

2.5. Total Flavonoid Content (TFC)

Total flavonoid content was measured by conducting an aluminum chloride colorimetric assay [34]. To a 100 µL of sample, 100 µL of a 2% solution of AlCl₃·6H₂O in methanol was added. The absorbance was measured after 10 min against methanol at 430 nm. Flavonoid concentration was expressed as milligrams of quercetin equivalents (QE) per gram dry weight of the sample (mg QE/g dry leaf).

2.6. Total Polyphenol Content (TPC)

TPC was measured using the method of Lee et al. [33]. Folin–Ciocalteu reagent (100 µL) was added to 100 µL of sample solution and reacted at room temperature for 3 min. After adding 100 µL of 2% sodium carbonate, the mixture was incubated at room temperature for 30 min. Absorbance was measured at 750 nm using an enzyme-linked immunosorbent assay (ELISA) reader with distilled water as the blank. Total phenolic content was reported as milligrams of gallic acid equivalents (GAE) per gram dry weight sample (mg GAE/g dry leaf).
2.7. Data Analysis

All the data were expressed as the mean ± standard deviation. Duncan’s multiple-range tests were used to detect statistically significant differences. Analysis of variance, correlation among phytochemicals and antioxidant activity, and Ward’s hierarchical clustering analyses were conducted using R statistical software [35]. The antioxidant capacity results obtained using the different chemical methods were used to calculate the RACI as in [19].

3. Results

3.1. Antioxidant Activity and Phytochemical Content

There were significant differences between the two years of the experiments and between genotypes, as well as genotype × year interactions, for DPPH and ABTS antioxidant activities, and for TFC and TPC contents (Figure 1 and Table 1). The antioxidant activity of DPPH of the 120 amaranth accessions ranged from 1.1 (accession A77) to 75.2 (A70) with an average of 30.8 mg AAE/g in 2018, and 8.5 (A63) to 68.8 (A92) with an average of 40.2 mg AAE/g in 2019 (Table 2 and Table S1). ABTS antioxidant activity ranged from 16.7 (A85) to 78.3 (A19) mg AAE/g (mean of 58.3) in 2018, and 56.6 (A80) to 79.2 (A89) mg AAE/g (mean of 66.4) in 2019. TFC averaged 37.4 mg QE/g in 2018, ranging from 21.7 (A53) to 52.7 (A111), and averaged 39.8 mg QE/g in 2019, ranging from 22.3 (A116) to 54.7 (A72). The mean TPC in 2018 was 94.1 mg GAE/g, ranging from 48.9 (A37) to 154.4 (A24), and was 111.7 mg GAE/g in 2019, ranging from 68.8 (A80) to 154.6 (A72).

![Figure 1](image_url)

This figure shows boxplots of DPPH, ABTS, TFC, and TPC in Amaranthus species cultivated in 2018 and 2019. * *** significant at \( p < 0.001 \).

| Genotype (G) | Year (Y) | Interaction (G × Y) |
|--------------|----------|---------------------|
| DPPH         | 11,376 *** | 185,186 *** | 6211 *** |
| ABTS         | 1324.7 *** | 23,968.5 *** | 620.9 *** |
| TFC          | 4253 ***   | 24,469 ***   | 1237 *** |
| TPC          | 1725.3 *** | 49,921.6 *** | 876.4 *** |

***, significant at \( p < 0.001 \).
Table 2. Descriptive statistics of antioxidant activities (DPPH and ABTS assays), total flavonoid content (TFC), and total polyphenol content (TPC) in 120 amaranth accessions.

| Year | Antioxidant Activity | Range   | Mean ± Standard deviation | CV (%) |
|------|----------------------|---------|---------------------------|--------|
| 2018 | DPPH (mg AAE/g)      | 1.1–75.2| 30.8 ± 17.6               | 57.1   |
|      | ABTS (mg AAE/g)      | 16.7–78.3| 58.3 ± 15.1              | 25.9   |
|      | TFC (mg QE/g)        | 21.7–52.7| 37.4 ± 6.6               | 17.6   |
|      | TPC (mg GAE/g)       | 48.9–154.4| 94.1 ± 24.2              | 25.7   |
| 2019 | DPPH (mg AAE/g)      | 8.5–68.8 | 40.2 ± 13.8               | 34.3   |
|      | ABTS (mg AAE/g)      | 36.6–79.2| 66.4 ± 9.5               | 14.3   |
|      | TFC (mg QE/g)        | 22.3–54.7 | 39.8 ± 6.1              | 15.3   |
|      | TPC (mg GAE/g)       | 68.8–154.6| 111.7 ± 19.4           | 17.4   |

1 Mean ± Standard deviation.

3.2. Relative Antioxidant Capacity Index

The accession A24 (1.92) had the highest RACI in 2018, followed by A70 (1.84), A72 (1.68), and A65 (1.65). A85 (−1.81) had the lowest RACI (Figure 2 and Table S1). In 2019, A72 (1.69) had the highest RACI, followed by A113 (1.61), A92 (1.40), and A91 (1.33), and A80 (−2.48) had the lowest. Accession A72 (1.68) had the highest RACI rank in total over both years, followed by A70 (1.46), A113 (1.38), and A92 (1.21). A116 (−1.99) had the lowest total RACI rank.

Figure 2. Relative antioxidant capacity index (RACI) of 120 Amaranth accessions. (A) RACI in 2018, (B) RACI in 2019, (C) total RACI.
### 3.3. Antioxidant Activities in *Amaranthus* Species

The antioxidant activities in leaves of the nine *Amaranthus* species grown in two years are given in Table 3. In 2018, DPPH ranged from 24.2 (A. crispus) to 49.9 (A. hybridus) mg AAE/g. Among the nine species, A. tricolor showed the most variation (CV = 75.2%) and A. hybridus showed the least variation (CV = 23.2%). ABTS ranged from 51.2 in A. caudatus to 69.9 mg AAE/g in A. blitum. Among the nine species, A. cruentus showed the most variation (CV = 33.8%) and A. hybridus the least (CV = 6.6%). In 2019, the DPPH of nine *Amaranthus* species ranged from 28.8 (A. tricolor) to 64.4 (A. dubius) mg AAE/g and ABTS ranged from 61.4 (A. tricolor) to 78.4 (A. dubius) mg AAE/g. Among the nine *Amaranthus* species, A. tricolor showed the most variation in DPPH (CV = 44.4%) and ABTS (CV = 18.9%).

#### Table 3. Descriptive statistics for antioxidant activities of nine *Amaranthus* species in 2018 and 2019.

| Year | Species | No. | DPPH (mg AAE/g) | ABTS (mg AAE/g) |
|------|---------|-----|----------------|-----------------|
|      |         |     | Mean ± SD     | CV (%)          | Mean ± SD     | CV (%)          |
|      |         |     |                |                 |                |                 |
| 2018 | A. blitum | 3 | 23.5±43.7 | 36.3±11.1 a² | 30.6 | 62.7±74.2 | 69.0±6.3 a b | 9.0 |
|      | A. caudatus | 18 | 6.1±48.7 | 25.5±16.6 ab | 63.1 | 26.9±77.6 | 51.8±14.7 b | 28.7 |
|      | A. crispus | 11 | 24.2±69.9 | 43.4±11.9 ab | 27.4 | 56.4±74.6 | 67.7±5.1 a b | 7.5 |
|      | A. cruentus | 7 | 3.5±53.6 | 24.2±17.8 ab | 73.6 | 25.6±75.8 | 54.2±18.3 a b | 33.8 |
|      | A. dubius | 6 | 10.9±42.1 | 26.9±15.3 ab | 56.9 | 44.2±74.0 | 59.7±15.1 ab | 25.3 |
|      | A. hybridus | 7 | 31.0±56.3 | 44.9±10.4 a b | 23.2 | 62.5±73.3 | 68.5±4.5 a b | 6.6 |
|      | A. hypochondriacus | 31 | 6.0±65.6 | 30.1±16.8 ab | 35.8 | 31.1±78.3 | 60.8±13.1 a b | 21.5 |
|      | A. tricolor | 30 | 11.1±75.2 | 27.6±20.9 ab | 75.7 | 16.7±78.1 | 53.3±17.9 a b | 33.6 |
|      | A. viridis | 7 | 9.0±53.2 | 35.3±15.4 ab | 43.6 | 28.7±74.0 | 60.9±15.2 ab | 25.0 |
| 2019 | A. blitum | 3 | 40.1±50.3 | 46.2±5.4 b | 11.7 | 72.9±75.2 | 74.9±11.5 ab | 1.8 |
|      | A. caudatus | 18 | 22.0±61.1 | 38.0±9.5 bc | 25.1 | 49.2±78.1 | 62.8±7.7 c | 12.3 |
|      | A. crispus | 11 | 26.5±55.5 | 41.6±8.5 ab | 20.3 | 58.4±73.6 | 69.7±4.5 ab | 6.5 |
|      | A. cruentus | 7 | 29.9±43.9 | 36.6±5.1 bc | 14.0 | 55.7±11.8 | 61.6±6.1 c | 9.9 |
|      | A. dubius | 6 | 60.1±68.8 | 64.4±3.4 a b | 5.3 | 77.5±79.2 | 78.4±1.0 a | 0.9 |
|      | A. hybridus | 7 | 33.7±66.5 | 45.5±11.3 ab | 24.8 | 68.9±7.8 | 73.5±3.0 ab | 4.1 |
|      | A. hypochondriacus | 31 | 19.6±67.4 | 45.5±11.0 ab | 24.2 | 45.7±73.1 | 68.1±7.7 bc | 11.3 |
|      | A. tricolor | 30 | 8.5±54.6 | 28.8±12.8 b c | 44.4 | 36.6±76.7 | 61.4±11.6 c | 18.9 |
|      | A. viridis | 7 | 10.3±56.6 | 44.5±19.2 b c | 43.1 | 43.7±77.4 | 69.1±11.5 ab | 16.6 |

² The same letter in each column indicates no significant difference by Duncan’s multiple range test, p < 0.05.

### 3.4. Total Flavonoid Content and Total Phenolic Content in *Amaranthus* Species

TFC and TPC in plants cultivated in 2018 and 2019 are shown in Table 4. The TFC ranged from 34.7 (A. cruentus) to 42.3 (A. tricolor) mg QE/g and 36.4 (A. tricolor) to 44.2 (A. blitum) mg QE/g in 2018 and 2019, respectively. The TPC of nine *Amaranthus* species ranged from 8.38 (A. cruentus) to 116.0 (A. blitum) mg GAE/g in 2018 and 100.1 (A. cruentus) to 141.9 (A. dubius) mg QE/g.

#### Table 4. Descriptive statistics for total flavonoid content (TFC) and total phenol content (TPC) of nine *Amaranthus* species cultivated in 2018 and 2019.

| Year | Species | No. | TFC (mg QE/g) | TPC (mg GAE/g) |
|------|---------|-----|---------------|---------------|
|      |         |     | Mean ± SD     | CV (%)        | Mean ± SD     | CV (%)        |
|      |         |     |                |               |                |               |
| 2018 | A. blitum | 3 | 35.0±44.1 | 38.1±5.2 ab² | 13.6 | 97.3±136.3 | 116.0±19.5 a | 16.8 |
|      | A. caudatus | 18 | 21.7±46.8 | 34.9±6.8 ab | 19.4 | 56.2±135.2 | 84.1±21.0 c | 25.0 |
|      | A. crispus | 11 | 35.1±48.0 | 40.4±4.2 ab | 10.3 | 96.2±137.6 | 117.0±14.1 a | 12.1 |
|      | A. cruentus | 7 | 27.5±47.1 | 37.1±7.5 ab | 20.3 | 48.9±125.2 | 83.8±28.3 c | 33.8 |
|      | A. dubius | 6 | 28.7±46.1 | 36.0±6.0 ab | 16.5 | 74.5±134.7 | 97.8±24.6 ab | 25.2 |
|      | A. hybridus | 7 | 34.4±52.7 | 42.3±5.4 a b | 12.8 | 103.3±126.1 | 113.0±7.9 ab | 7.0 |
|      | A. hypochondriacus | 31 | 23.9±51.1 | 39.3±5.3 b c | 13.6 | 52.5±154.4 | 90.8±25.0 bc | 27.5 |
|      | A. tricolor | 30 | 24.9±49.9 | 34.7±7.3 b c | 21.1 | 53.5±146.8 | 89.0±24.1 bc | 27.1 |
|      | A. viridis | 7 | 23.6±44.1 | 37.4±7.0 b c | 18.7 | 61.7±113.8 | 98.8±18.1 abc | 18.3 |

² The same letter in each column indicates no significant difference by Duncan’s multiple range test, p < 0.05.
3.5. Correlations among Antioxidant Activities, Total Phenolic Content, and Total Flavonoid Content

In the accessions grown in 2018, there were significant positive correlations ($p < 0.05$) between DPPH and ABTS ($r = 0.89$), TPC and TFC ($r = 0.74$), DPPH and TFC ($r = 0.77$), DPPH and TPC ($r = 0.92$), ABTS and TFC ($r = 0.77$), and ABTS and TPC ($r = 0.88$) (Figure 3A). In the accessions grown in 2019, there were significant positive correlations ($p < 0.05$) between DPPH and ABTS ($r = 0.87$), TPC and TFC ($r = 0.80$), DPPH and TFC ($r = 0.74$), DPPH and TPC ($r = 0.79$), ABTS and TFC ($r = -0.79$), and ABTS and TPC ($r = 0.76$) (Figure 3B). For each assay over both years, there were significantly positive correlation ($p < 0.05$) between DPPH and ABTS ($r = 0.91$), TPC and TFC ($r = 0.78$), DPPH and TFC ($r = 0.81$), DPPH and TPC ($r = 0.89$), ABTS and TFC ($r = 0.81$), and ABTS and TPC ($r = 0.90$) (Figure 3C).

![Figure 3](image-url)

**Figure 3.** Correlation coefficients between antioxidant activities (DPPH and ABTS) and phytochemicals (TPC and TFC). (A) 2018, (B) 2019, and (C) 2018 and 2019 combined.

3.6. Hierarchical Clustering Analysis

The results of hierarchical clustering analysis, using the Ward’s method between groups, are shown in Figure 4 and Table 5. We classified 120 amaranth accessions into three groups according to their antioxidant activities, TFC, and TPC in each cultivation year (Figure 4A). Cluster I contained 55 accessions and had higher TFC, TPC, and antioxidant activities in both years than the other clusters. Cluster II consisted of 42 accessions and had high TFC, TPC, and antioxidant activities in 2019, while these levels grouped between clusters I and III in 2018. Cluster III included 23 accessions, and had the lowest TFC, TFC, and antioxidant activities in both of the years.

The nine *Amaranthus* species also divided into three clusters (Figure 4B). *A. blitum*, *A. hybridus*, and *A. crispus* had higher TFC, TPC, and antioxidant activities in the two years than the other species. *A. dubius*, *A. viridis*, and *A. hypochondriacus* had higher TFC, TPC, and antioxidant activities in 2019 and intermediate levels in 2018. *A. cruentus*, *A. caudatus*, and *A. tricolor* had lower TFC, TPC, and antioxidant activities in both years than the other species.
Figure 4. Hierarchical clustering of antioxidant activity and phytochemical content in *Amaranthus* species cultivated in 2018 and 2019. (A) Hierarchical clustering of measurements from 120 *Amaranthus* accessions. (B) Hierarchical clustering of measurements from nine *Amaranthus* species. Blue, lower value of each parameter; Red, higher value of each parameter.

Table 5. Average cluster values (±standard deviation, SD) of antioxidant activities, total flavonoid content, and total polyphenol content of 120 Amaranth accessions.

| Year | Group | No. acc | DPPH (mg AAE/g) | ABTS (mg AAE/g) | TFC (mg/QE/g) | TPC (mg/GAE/g) |
|------|-------|---------|----------------|----------------|---------------|----------------|
|      |       |         |                |                |               |                |
| 2018 | I     | 55      | 46.1 ± 12.2 a  | 70.8 ± 4.9 a   | 41.7 ± 5.0 a  | 115.4 ± 14.7 a |
|      | II    | 42      | 21.1 ± 8.3 b   | 53.5 ± 8.2 b   | 36.5 ± 4.1 b  | 80.1 ± 13.0 b  |
|      | III   | 23      | 12.0 ± 8.4 c   | 37.2 ± 12.5 c  | 28.6 ± 3.4 c  | 68.9 ± 12.8 c  |
| 2019 | I     | 55      | 45.4 ± 11.1 a  | 70.7 ± 6.3 a   | 42.5 ± 4.5 a  | 120.4 ± 15.5 a |
|      | II    | 42      | 42.5 ± 11.5 a  | 67.9 ± 6.2 a   | 40.9 ± 4.1 a  | 112.8 ± 17.3 b |
|      | III   | 23      | 23.7 ± 11.3 b  | 53.6 ± 9.9 b   | 31.5 ± 5.7 b  | 88.9 ± 12.5 c  |

1 The same letter in each column indicates no significant difference by Duncan’s multiple range test, *p* < 0.05.
4. Discussion

Many previous studies evaluated antioxidant activity [4–8,25,36–38] and phytochemicals content of various *Amaranthus* species [8,23–25,36,38–40]. For instance, Tatiya et al. reported that TPC and TFC of *A. spinosus* and *A. viridis* were 43.4 ugGAE/g fresh weight (FW) and 177.6 rutin equivalent (RE) ug/g dried weight (DW) and 25.7 43.4 ugGAE/g FW and 179.1 RE ug/g DW, respectively [38]. In addition, DPPH and ABTS of *A. spinosus* and *A. viridis* showed 23.5 and 26.6 trolox equivalent antioxidant capacity (TEAC) ug/g DW and 48.4 and 50.0 TEAC ug/g DW, respectively [39]. Li et al. reported that TPC and TFC of leaves extracts in *A. hypochondriacus* and *A. caudatus* showed 14.9 and 10.5 mg GAE/g DW and 11.4 and 6.5 mg CAE/g DW, respectively. They also determined antioxidant activities of two *Amaranthus* species using FRAP (Ferric Reducing Antioxidant Power) and ORAC (Oxygen Radical Absorbance Capacity) [24]. Pamela et al. reported that TPC and TFC of *A. Caudatus*, *A. Cruentus*, *A. Hybrid*, *A. Hypochondriacus* and *A. Hybridus* were 27.5, 30.5, 29.7, 30.0, and 30.8 mg GAE/100 g and 8.9, 9.9, 9.0, 9.5, and 9.6 mg CE/100 g, respectively. They also determined antioxidant activities of five *Amaranthus* species using various method, such as DPPH, FRAP, ABTS [40]. Our study reveals variation amongst the 120 accessions of nine *Amaranthus* species for antioxidant activities and phytochemicals content. However, our results could not be compared with the previous studies because of their different methods such as different standard materials (ex. Quercetin of TFC in our study vs. Rutin in Tativa et al. [38] vs. catechin in Pamela et al. [40]). The Aamaranthaceae family approximately consists of 70 Amaranthus species, of which 20 produce edible leaves and/or grains [39]. We evaluated nine of these for their potential to serve as sources of antioxidants in food or for medicine. The variation we identified can serve as basic information for the use of *Amaranthus* species and accessions as breeding material.

Many methods of measuring antioxidant activity have been developed to determine the antioxidant capacity of plant extracts. Those that use relatively standard equipment and can deliver fast and reproducible results are the most useful [34]. In this study, antioxidant activities were evaluated using both the DPPH and ABTS methods. Both assays are based on electron transfer [41,42] and, in our study, DPPH scavenging activity correlated well with ABTS scavenging activity (Figures 1 and 3).

In this study, TPC, TFC, DPPH, and ABTS showed strong positive correlations among them (Figure 1). In general, TPC and TFC are chain breakers, free radical scavengers, or electron donors that contribute to antioxidant activity [43]. Polyphenols significantly reduce ROS/reactive nitrogen species, such as OH$^-$, O$_2^-$, NO$^-$, or OONO$^-$, preventing damage to biomolecules or formation of more reactive ROS [44,45]. The antioxidant activity of polyphenols depends on the structure of their functional groups. The number of hydroxyl groups influences mechanisms such as radical scavenging and metal ion chelation ability [46]. Among polyphenols, flavonoids, well known as antioxidant agents, are direct scavengers of free radicals, resulting in more stable, less-reactive radical species [47,48].

We identified significant differences in the both contents of polyphenols and flavonoids and in antioxidant activities between plants grown in 2018 and 2019 (Figure 1). TPC, TFC, and antioxidant activities were all higher in plants grown in 2019 than in 2018. The differences between years could be explained by climate variations. During the 2019 growing season, there was 53.3% less rainfall than in 2018 (388.1 mm compared with 728.1 mm during May to August), while the average and accumulated temperatures were similar between 2018 (25 °C and 3079.7 °C, respectively) and 2019 (24.2 °C and 2986.1 °C, respectively) [49]. Sunshine is thought to boost phytochemical contents, because photosynthetic performance influences polyphenol synthesis [30]. Environmental differences cause differences in TPC, which then affect antioxidant activity [11]. The results of our study also suggest that photosynthetic performance in 2019, when the rainfall was low, would have been higher than in 2018, which would have led to higher TPC and TFC. The differences in TPC and TFC between plants grown in the two years would then have affected antioxidant activity.
In hierarchical clustering analysis, 42 *Amaranthus* accessions in cluster II (Figure 4A) belonging to three species, *A. dubius*, *A. viridis*, and *A. hypochondriacus* (Figure 4B), had significantly different TFC, TPC, and antioxidant activities between the two years compared with other accessions or species groupings. Genotype environment interactions [10] in plants grown in suboptimal or super-optimal environments reflect how sensitive the plants are to the environment. The response of genotypes grown in suboptimal conditions reflects different efficiencies of the genotype, while differences in super-optimal environments reflect the different tolerances of the genotypes [51]. Variation in gene expression has a major effect on the phenotypic variation within species [52] and the differentiation among species and gene expression, or the responsiveness of expression to environmental stimuli, can vary among species. Our results suggest that the 42 *Amaranthus* accessions and/or three species that differed significantly in antioxidant activities and phytochemical content between the two cultivation years were more sensitive to the environment than were the other accessions and/or species.

*Amaranth* is ranked as having one of the top five antioxidant capacities across vegetable crops [53]. *Amaranthus* species are divided into grain species (*A. hypochondriacus*, *A. caudatus*, and *A. cruentus*), vegetable species (*A. tricolor* L., *A. blitum* L., *A. dubius*, and *A. viridis*), and weed species (*A. hybridus* and *A. crispus*) [2]. We established that phytochemical (TFC and TPC) contents and antioxidant activities were lower in the grain species (*A. caudatus*, *A. cruentus*, and *A. hypochondriacus*) than in the other groups of *Amaranthus*, and two weed species had higher phytochemical contents and antioxidant activities (Tables 2 and 3, and Figure 4B). In general, the leaves of vegetable *Amaranthus* species are rich in phytochemicals such as lysine-rich protein, β-carotene, various vitamins, and minerals and dietary fiber [2]. The seeds of grain *Amaranthus* species have been shown to contain squalene, trypsin inhibitor, tocotrienols, and tannins, but previously there has been little research into their leaf composition. Likewise, there has not been much prior research conducted into the phytochemistry or biological activity of antioxidants in the weed species of *Amaranthus*.

5. Conclusions

The results of this study highlight the potential of 120 *Amaranthus* accessions from nine species to be used as sources of phytochemicals and antioxidants. *Amaranthus* sp. is an underutilized and ignored plant despite its high protein and nutrients content. However, the latest studies underline the beneficial impact of hydrolysates and bioactive peptides of *Amaranthus* sp. as free radical scavengers and mainly influenced by peptide molecular weight, structure, and its amino acid composition [44]. Compared to previous studies, although the Amaranthaceae family contains approximately 70 *Amaranthus* species, of which 20 produce edible leaves and/or grains [39], we considered that our results were a more detailed evaluation of phytochemical content and antioxidant activity using various species and environmental effects for new utilization possibilities of *Amaranthus* sp. In particular, by finding higher phytochemicals content and antioxidant activity on weed species (*A. hybridus* and *A. crispus*) we were able to identify the potential for new health benefit materials. The results of this study provide basic information that can guide decisions in breeding programs using *Amaranthus* leaves.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11061032/s1, Table S1: List of 120 Amaranthus accessions in this study.

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